The environmental toxicology of zinc oxide nanoparticles to the oligochaete *Lumbriculus variegatus*

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Abstract

This thesis investigated the potential toxicity of zinc oxide nanoparticles (NPs) and bulk particles (both with and without organic matter (HA)) to the Californian Blackworm, *Lumbriculus variegatus*. The NPs and bulk particles in this thesis were characterised using numerous techniques. ZnO NPs were found to be 91 ($^{133}_{64}$) nm (median (interquartile range)) and ZnO bulk particles were found to be 237 ($^{322}_{165}$) nm (median (interquartile range)) by TEM.

In the acute behavioural study (96 hour), ZnO NPs had a dose-dependent toxic effect on the behaviour of the worms up to 10mg/L whereas the bulk had no significant effect. This result, however, was mitigated by the addition of 5mg/L HA in the NP study whereas a similar addition enhanced the toxicity of the bulk particles at 5mg/L ZnO. In the chronic study (28 days), ZnO NPs and bulk particles were found to have a dose-dependent significant effect on the behaviour of the worms after 28 days, with NPs causing a significantly greater negative response than bulk particles at 12.5, 25 and 50mg/L ZnO. HA had no effect on the toxicity of either particle type in the chronic study.

Acute (96 hour) oxidative stress in *L. variegatus* in response to ZnO NP and bulk particle (with and without 5mg/L HA) exposure was evaluated by examining the changes in glutathione (GSH) content of cells, since NPs are expected to have potential for toxicity via mechanisms such as oxidative stress. Neither ZnO NPs nor bulk particles (with and without 5mg/L HA) were found to induce significant changes in the GSH content of *L. variegatus* cells after 96 hours of exposure.

The uptake, accumulation and depuration of ZnO NPs and bulk particles in *L. variegatus* over a 48 hour period of uptake and a 48 hour period of depuration were also investigated. This study found a high level of variability and it was concluded that the protocol employed was not suitable for investigating the uptake, bioaccumulation and depuration of ZnO NPs and bulk particles.

Finally, histological techniques and a number of fixatives were evaluated for use with *L. variegatus*. Bouin's solution was found the most suitable fixative for use with these worms, with no histological damage observed in the morphology of the worms after a 96 hour exposure to ZnO NPs and bulk particles (with and without 5mg/L HA).

When considering the toxicology results from all experiments within this thesis it is concluded that ZnO NPs can cause both acute and chronic toxicity in terms of behavioural response, but do not cause acute oxidative stress in *L. variegatus*.

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Glossary

Acid phosphatase (AcP)

Agency for Toxic Substances and Disease Registry (ATSDR)

Analysis of variance (ANOVA)

Atomic Force Microscopy (AFM)

Bisphenol A (BPA)

British Standards Institute (BSI)

Brunauer, Emmett and Teller Method for surface area analysis (BET)

Carbon black NPs (CB)

Catalase (CAT)

Deionised water (DI water)

Dry weight (DW)

The Technical University of Denmark (DTU)

Derjaguin, Verwey, Landau and Overbeek (DVLO)

Deoxyribonucleic acid (DNA)

Dynamic Light Scattering (DLS)

Electrical double layer (EDL)

Energy Dispersive X-ray Spectroscopy (EDX)

Ethylenediaminetetraacetic acid (EDTA)

Facility for Environmental Nanoscience Analysis and Characterisation (FENAC)

General linear model (GLM)

Glutathione (GSH)

Glutathione disulphide (GSSG)

Glutathione peroxidise (GPX)

Glutathione-S-transferase (GST)

Haematoxylin and eosin stain (H and E)

Heriot Watt University (HWU)

Hydrodynamic Data (HD)

Inductively coupled plasma atomic absorption spectrophotometry (ICP-AAS)

Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Industrial methylated spirit alcohol (IMS)

International Organisation for Standardisation (ISO)

Kilo counts per second (kcps)

Lethal concentration at which 50% of the test community are affected (LC_{50})

Lipid peroxidation (LPO)

Molecular weight cut off (MWCO)

N-acetyl-hexozaminidese (HEX)

Nanomaterials (NMs)

Nanoparticles (NPs)

Natural Environment Research Council (NERC)

Natural organic matter (NOM)

Nicotinamide adenine dinucleotide phosphate (NADPH)

No effect concentration (NOEC)

Null hypothesis (NH)

o-Phthalaldehyde (OPT)

Organisation for Economic Co-operation and Development (OECD)

Perfluorooctane sulfonate (PFOS)

Polydispersity index (PdI)

Polychlorinated biphenyl (PCB)

Polycyclic aromatic hydrocarbon (PAH)

Polyvinyl acetate (PVA)

Publicly Available Standard (PAS)

Reactive oxygen species (ROS)

Redox Quenching Buffer (RQB)

Scanning Electron Microscopy (SEM)

Scanning Transmission Electron Microscopy (STEM)

Soluble glutathione-S-transferase (sGST)

Standard deviation (SD)

Standard error (SE)

Standard operating procedure (SOP)

Superoxide dismustase (SOD)

Suwannee River humic acid (HA)

Technical University of Denmark (DTU)

Transmission Electron Microscopy (TEM)

United States Environmental Protection Agency (USEPA)

United States Environmental Protection Agency Reconstituted Hard Water Medium (EPA HW)

Wet weight (WW)

X-Ray diffraction (XRD)

Zeta potential (ZP)

β-glucuranidase (β-GUS)

2-ethyl-hexyl-4-trimethoxycinnamate (EHMC)

Chapter 1 Introduction

The concept of nanoscale technology came about with the visionary and historical lecture given by Richard Feynman on the 29th December 1959 at the annual American Physical Society. The lecture titled "There's Plenty of Room at the Bottom: Invitation to Enter a New Field of Physics" sparked intrigue and excitement from his peers as he investigated "manipulating things on a small scale" and how "ultimately – in the great future – we can arrange atoms the way we want; the very atoms, all the way down!" (Roduner, 2006; Hunt and Mehta, 2006). Feynman suggested writing the entire twenty four volumes of the Encyclopaedia Britannica on the head of a pin and also suggested that the electron microscope be made 100 times better. He gained much of his inspiration from biology as he understood that information is stored on a molecular level and that cells manufacture substances and operate on a very small scale (Roduner, 2006).

1.1 Nano definitions

Nanoparticles (NPs) are defined as particles with all three external dimensions in the nanoscale (1 – 100nm) (Publicly Available Standard (PAS) 136; BSI 2007) and nanoscience refers to the study of the fundamental principles of molecules and structures with at least one dimension roughly between 1 and 100nm (Ratner and Ratner, 2003). These structures are known as nanostructures (Ratner and Ratner, 2003) and the application and manipulation of these nanostructures is known as nanotechnology (Ratner and Ratner, 2003). At the most fundamental level in nanoscale, size has a great effect on their properties, an effect that is unseen at any other level. This coupling of size with the most fundamental chemical, electrical and physical properties of materials is key to nanoscience (Ratner and Ratner, 2003). NPs have a larger surface area to volume ratio than their bulk counterparts allowing for greater reactivity and they also possess certain physicochemical properties that allow them to have very different characteristics to those particles of larger size (MacCormack and Goss, 2008). NPs are specifically engineered to exploit these electrical, thermal, mechanical and imaging properties that are lacking in their larger counterparts (Liu, 2006). Nanomaterials (NMs) are defined as a material which contains single or agglomerated/aggregated particles where a minimum of 50% of those particles have at

least one dimension in the scale of 1 – 100nm (European Commission, 2011/696/EU, 2011). Aggregation of nanoparticles occurs when particles are bonded strongly to each other and the total surface area of the aggregate is reduced (ISO TS 27687, 2008). Agglomeration occurs when nanoparticles or aggregates are weakly bonded together and the total surface area of the agglomerate is similar to the sum of the surface areas of the individual particles/aggregates (ISO TS 27687, 2008).

1.2 Nanoparticles and the environment

Since the 1990s there has been a rapid increase in both the use of engineered NPs and the implementation of nanotechnologies. According to the Woodrow Wilson Project on Emerging Nanotechnologies (http://www.nanotechproject.org/, 10/09/2012) there are 1317 products currently on the world market that are classed as nanotechnology products (either NM-containing or products that have been produced using nanotechnology). Since the inventory was first released in 2006 it has grown by almost 521%. The main category of products falls under health and fitness, which include items such as sunscreens and cosmetics. The inventory has listed 30 countries of origin for these products with the main supplier of nanotechnology products being the United States, followed by Europe and East Asia (respectively) and elsewhere around the world. The most common materials are (in order of most to the least common) silver, carbon, titanium (including TiO_2), zinc (including ZnO) and gold. One of the side effects of this "nano-boom" is the potential for these NPs and NMs to be released to the environment (Bystrzejewska-Piotrowska *et al.*, 2009). Due to this potential there is an urgent need for research programmes in the field of nanotoxicology. Researchers have expressed interest in both the positive and negative effects of nanotechnology. Advocates point towards benefits like better medicines, improved consumer products and environmental remediation, however others have expressed concern over the lack of available knowledge on how these NPs function, what their fate may be in the environment and the potential they may have to stress the environment or human systems (Klaine *et al.*, 2011). In recent years the Organisation for Economic Co-operation and Development (OECD) launched an initiative to test the human and environmental impacts of the engineered NPs that are already in use (http://www.oecd.org, 25/08/2012). The OECD assists countries to implement policies

to ensure the safe development of nanotechnologies. The programme sought to ensure that the approach to hazard, exposure and risk assessment was of a high, science based and internationally harmonised standard. Experts in the field suggested that this initiative should include both NPs/NMs that were already on the market and those that may be developed in the future (Trager, 2008). Attention is currently focusing on a large number of engineered NPs, including fullerenes, carbon nanotubes, quantum dots and nanoscale metal oxides, amongst others, that are beginning to reach the market in growing quantities and in a wide variety of applications (www.nanotechproject.org, 2012). In order to examine the environmental risks associated with NP exposure, research must identify and quantify the source, determine the release pattern of the nanomaterials, establish the concentrations present in the environment and examine the potential of the nanomaterials to bioaccumulate (SCENIHR, 2006). NPs have always existed from both natural (e.g. volcanic ash, combustion) and anthropogenic (e.g. car exhaust, engineered NPs, cigarette smoke) sources and so there is a natural background concentration of NPs in the environment. (Natural NPs are NPs that are formed during natural processes (e.g. volcanic ash), incidental NPs are formed as a by-product of natural or manmade processes (e.g. welding, mining, combustion) and manufactured NPs are particles that have been engineered for a specific property or composition (e.g. carbon nanotubes, metal oxide NPs) (PAS 71, BSI, 2005)). It has been suggested however that this background concentration may be low in comparison to the potential releases of engineered NPs to the environment (Klaine *et al.*, 2008). NPs can enter the environment through a number of routes including both intentional and unintentional releases. Unintentional releases can result from atmospheric emissions, solid or liquid waste streams from industrial facilities or transport accidents and spillages. They will also enter the environment from cosmetics, paints, sunscreens, fabrics, food packaging and medical products in relation to the use of these products in society. Deliberate releases include those of remediation attempts (e.g. Fe NPs – Zhang & Elliot, 2006). The physicochemical properties of the NPs will determine their bioavailability and toxicity (Powers *et al.*, 2006). The physical and chemical properties of the receiving environment will play a critical role in determining the fate of the particle in the environment and this will affect the behaviour and toxicity of the particle (USEPA, 2007). NPs are also prone to aggregation/agglomeration and sorption onto organic and inorganic material and so this will also change their fate in the environment (Holsapple *et al.*, 2005). Another point to consider is that NPs are not always released to the

environment as single NPs. In many applications, NPs are embedded in a matrix or functionalised with coatings, e.g. NPs used in biomedicine. These matrices and coatings can also be affected by organic and inorganic matter, pH, salinity and the ionic content of the medium they are in and this may result in the modification of the NP and may in turn release a free NP to the environment (Norwalk and Bucheli, 2007). Due to the different compositions of NP batches and aggregates/agglomerates, their potential toxicity can be increased or decreased and their behaviour in the environment can be varied and difficult to determine.

Estimates of NPs present in surface waters and other media have been provided in the literature based on predicted use, exposure scenarios and modelling (Boxhall *et al*., 2007; Mueller and Nowack, 2008; Gottschalk *et* al., 2010; Nowack *et al*., 2012) but these approaches are not sufficient to provide quantitative estimates of NP concentrations in the environment. While techniques for the detection, identification and quantification of NPs in the environment are being developed, it remains a challenge to monitor engineered NPs in the environment (ENRHES, 2010). Part of this gap has resulted due to a lack of funding into this area (Klaine *et al.*, 2011). For example, according to the National Nanotechnology Initiative (http://www.nano.gov, 01/10/2012), between 2005 and 2011, the U.S spent almost $10 billion on nanotechnology research. During the same period only $480 million was spent on understanding the potential impact of this technology on the environment and health and safety issues. The aquatic environment is thought to be where nanomaterials may end up since it ultimately receives run off and wastewater inputs from both domestic and industrial sources and so the investigation into the effects of NPs/NMs on the aquatic environment (both water column and sediment) is of utmost importance (Baun *et al*., 2008).

1.3 Zinc

For both humans and the environment, zinc is an essential micronutrient (WHO, 2001). Zinc is found throughout the environment and constitutes 20 – 200ppm (by weight) of the Earth's crust. Because of its reactivity it is mainly found as zinc oxide (ZnO) or sphalerite (ZnS) (ATSDR, 2005). Since zinc is an essential element for all organisms, including humans, it is implied that there will be a minimum amount of zinc which will

supply the needs of the organism but also a maximum amount above which zinc will become toxic (Hopkins, 1993). The range between the minimum and maximum concentrations is often referred to as the "window of essentiality" (Hopkins, 1993). The background concentration of zinc in an ecosystem is partly bioavailable. This partial bioavailability provides organisms in that ecosystem with sufficient essential metals and so contributes to biodiversity under normal conditions.

1.3.1. Zinc speciation and solubility

As well as being an essential element and present naturally in ecosystems, the speciation of zinc in the environment may be very relevant for biological processes and thus, for potential risks (Bodar *et al*., 2005). In both fresh and seawater, zinc exists in particulate and dissolved forms and is distributed over a number of chemical species (Bodar *et al*., 2005). Zinc does not degrade in the environment but can change from one form to another, both reversibly and irreversibly, in a number of chemical reactions and under numerous environmental conditions (ATSDR, 2005). A number of metals are known to form ions when in aqueous media and the toxicity of these ions has been well documented (Campbell, 1995). Based on their bulk counterparts the solubility of ZnO NPs may play an important role in their toxicity (Bai *et al*., 2009). It has been observed that material solubility strongly influences the cytotoxic response of a number of metal oxide nanoparticles, with more soluble compounds like ZnO and Fe_2O_3 showing greater acute toxicity than NPs of low solubility such as CeO_2 and TiO_2 (Brunner *et al*., 2006). Aquatic organisms can be very sensitive to dissolved zinc and so understanding the dissolution behaviour of ZnO is essential in order to avoid a potential misinterpretation of results (Franklin *et al*., 2007). Zinc can occur in both suspended free ions and dissolved complex or compound forms in water (ATSDR, 2005). The conditions of the water body (e.g. pH, organic/inorganic content, solution speciation etc.) will affect metal NP dissolution and stability (Johnston *et al*., 2010). In water, zinc most often occurs in the 2^+ oxidation state and it dissolves in acid conditions to form hydrated Zn^{2+} cations and in strong basic conditions to form zincate anions (O'Neil *et al*., 2001). It often forms complexes in the presence of organic and inorganic matter (USEPA, 1979; 1984, 1987). In natural environments, complexing agents, such as humic acid, can bind zinc (Guy and Chakrabarti, 1976). The stability of these complexes is dependent on the

pH of the surrounding waters (Guy and Chakrabarti 1976). As the pH decreases the concentration of Zn ions in the water phase can increase (ATSDR, 2005). In sediments, zinc can adsorb onto hydrous iron and manganese oxides, clay minerals and organic material. In addition to this the conditions of the sediment, such as depth, organic content and clay content, can affect how the zinc behaves (ATSDR, 2005).

1.3.2 ZnO nanoparticles and their toxicity

Zinc oxide NPs have a variety of applications, such as optoelectronics, cosmetics, catalysts, ceramics, pigments (Bai *et al*., 2010) and personal care products (Blinova et al., 2010), due to their unique properties and diverse nanostructures. Annually, on a world-wide scale, a million tonnes of ZnO are produced. The properties of ZnO, e.g. UV absorption and specific surface area, are improved at the nanoscale. ZnO NPs have been shown to be more efficient at absorbing UVA radiation than TiO_2 NPs (Pinnell *et al*., 2000) and ZnO NPs are also transparent which is advantageous to the cosmetics and sunscreen industries (Houdy *et al*., 2011). Of the 1317 products previously mentioned 24 were found to use zinc oxide nanoparticles. Products on the Woodrow Wilson website include sunscreens, moisturisers, make up, lipsticks and self cleaning coatings, however this is not a fully comprehensive list. Natural zinc NPs exist in ecosystems and play an important role in biogeochemical processes as zinc is an essential micronutrient (Wigginton *et al*., 2007), however the potential impact of engineered ZnO NPs on the environment has yet to be fully evaluated. Recent studies have suggested that ZnO NPs can have adverse effects on human and environmental health. ZnO NPs are recognised as a respiratory toxicant (i.e. they caused an inflammatory response in the lungs) which resulted in metal fume fever (Beckett *et al*., 2005) and it has been shown that ZnO NPs cause oxidative stress and an inflammation response in vascular/lung endothelial cells (Gojova *et al*., 2007; Lin *et al*., 2009). Research has also indicated that the liver, spleen, heart, pancreas and bone were all targets for pathological damage of orally administered ZnO nanoparticles in the 20 -120nm range (Wang *et al*., 2008).

Ecotoxicological studies have indicated that ZnO NPs can cause adverse effects in a number of species. Lin & Xing (2008) investigated the toxicity of ZnO NPs and

Zn^{2+} ions to ryegrass seedlings in bulk nutrient and rhizosphere solution. A concentration dependent decrease in seedling biomass was observed for both ZnO nanoparticles and Zn^{2+} treated ryegrass and the 50% biomass inhibitory concentrations (LC_{50}) were estimated to be 64mg/L for ZnO nanoparticles and 38mg/L for Zn^{2+}. Aruoja *et al*. (2008) conducted growth studies using *Pseudokirchneriella subcapitata* (green algae) using ZnO NPs. EC_{50} values were found to be similar in both ZnO NPs and bulk particles and toxicity was attributed to Zn^{2+}. Franklin *et al*. (2007) compared the toxicity of sonicated ZnO NPs and sonicated ZnO NPs in the presence of a surfactant on *Pseudokirchneriella subcapitata*. The observed lethality could be attributed to dissolved Zn(II). Mortimer *et al*. (2008) performed toxicity tests on V*ibrio fischeri* (bacteria) using a flash assay (cuvettes and microplates) and microtox tests. The flash assay and microtox assay are both luminescence based assays which takes into account the colour and turbidity of a sample (Heinlann *et al*., 2008). The 30-min EC_{50} values were reported to be 4.8 ± 1.1 and 3.9 ± 1.8 mg/L in cuvettes and microplates, respectively. No toxic effect was observed for ZnO in Microtox testing at the maximum concentration tested. Huang *et al*. (2008) reported a concentration-dependent decrease in the number of *Streptococcus agalactiae* (bacteria) and *Staphylococcus aureus* (bacteria) colonies after overnight incubation at concentrations of up to 0.12M (9.77g/L) PVA-coated ZnO NPs. A concentration of 0.12M (9.77g/L) ZnO caused more than 95% inhibition of bacterial growth, whereas concentrations of 0.0012 (97.69mg/L) and 0.006M (488.46mg/L) ZnO caused about 30% inhibition. Damage and integrity loss in *Streptococcus agalactiae* cells walls as well as cell penetration and membrane damage was also observed. Adams *et al*. (2006) studied the effects of ZnO on *Bacillus subtilis* (bacteria) and *Escherichia coli* (bacteria) at concentrations of 10 – 5000ppm and overnight incubation. The study found that the antibacterial effects of the NPs increased with increasing particle concentration. Blinova *et al*. (2010) reported the effects of ZnO nanoparticles on *Daphnia magna* (Water Flea), *Thamnocephalus platyurus* (Fairy Shrimp) and *Tetrahymena thermophila* (protozoa) in artificial freshwater and the 48 hour EC_{50} were found to be 2.6 ± 1.04mg/L, 0.14 ± 0.02mg/L and 9.4 ± 3.0mg/L, respectively. Adams *et al*. (2006) reported the effects of ZnO nanoparticles on *D. magna* at concentrations of 0.2 – 1mg/L over 8 days. No concentration-response relationships were established. Heinlaan *et al*. (2008) reported the effects on ZnO nanpoarticles on *D. magna* and found the $LC_{50\ 48hr}$ value to be 3.2 ± 1.3 mg/L and the NOEC value was found to be 0.5mg/L nominal

concentration. The $LC_{50\ 24hr}$ value for *Thamnocephalus platyurus* was found to be 0.18 ± 0.03 mg/L and the NOEC value was found to be 0.05mg/L for ZnO. Zhu *et al*. (2008) reported on the toxicity of ZnO NPs in *Danio rerio* (zebra fish) embryos and larvae. This study observed a 96 hour dose-dependent decrease in survival rates after exposure to ZnO NP concentrations of 0, 0.1, 0.5, 1, 5 and 50mg/L. The ZnO formed irregularly shaped aggregates in the water suspensions during these tests. In concentrations ≤ 0.5mg/L no toxicity was observed in larvae or embryos of zebra fish, whereas no survival was seen at 50mg/L. A similar does-dependent relationship as observed for bulk ZnO.

The findings of the literature review for ZnO NPs suggests that it is a highly toxic NP *in vitro* and in many environmental studies, and oxidative stress and solubility as well as ion toxicity play a major role in its mechanism of action. The range of ZnO NP concentrations varies quite a lot across the studies and the results are species specific. A number of environmental studies found no or little differences between toxicity of micro- and nano-scale ZnO particles. Development and reproduction were affected, although, these findings were not universal, whereas some organisms showed clear nano-scale effects, where the toxicity of NPs exceeded that of micro-scale particles and equivalent concentrations of zinc ions. There appears, however, to be a significant gap in the literature dealing with the effects of ZnO NPs on sediment dwelling organisms, such as *L. variegatus*.

1.4 *Lumbriculus variegatus*

1.4.1 Biology and Ecology

Lumbriculus variegatus (Californian Blackworm or Mudworm) is a member of the Lumbriculid family of fresh water oligochaetes. They are found throughout North America and Europe (Brinkhurst & Jamieson, 1971) and have been introduced to the southern hemisphere (Yamaguchi, 1953; Cook, 1971). *L. variegatus* was first described by Müller in 1774. The head segments of *L. variegatus* are more darkly pigmented than the tail section and are also more manoeuvrable than the tail segments. The first 8-10 segments include a conical prostomium, muscular pharynx and both male and female sex organs. Unlike other oligochaetes, *Lumbriculus* species do not have fixed regional

and numerical identities for their segments once they have matured (Lesiuk and Drewes, 2001). Locomotive behaviours in *L. variegatus* also make them excellent test species for toxicity testing. These behaviours are context specific and are highly stereotyped and so are ideal for investigating the sublethal effects of toxic substances (Ding *et al*., 2001). In sediments, touch or shadows will evoke an escape reflex, where the worm will rapidly withdraw into the sediment. On wet surfaces the worms will crawl forward or backward when touched and in water the worm will respond to a touch stimulus by helical swimming or body reversal movements (Drewes, 1999). These behaviours are discussed in greater detail in Chapter 3, Section, 3.1.1.

Oligochaetes are important constituents of freshwater ecosystems as they feed on subsurface sediments, processing and recycling deposited material (Leppänen and Kukkonen, 1998). In areas where there is a high density of worms, reworking and recycling of the sediment can substantially restructure the sediment itself (Krezoski and Robbins, 1985; McCall and Fisher, 1980). The sediment reworking and recycling by oligochaetes have been documented to cause significant changes to the biological, chemical and physical characteristics of sediments and overlying waters (McCall and Tevesz, 1982). *L. variegatus* has been reported to occur at densities of up to 11,000 worms/m^2 (Cook, 1969), in a wide variety of aquatic habitats, including streams (Healy, 1987), marshes (Verdonschot, 1999) and man-made water bodies (Bailey and Liu, 1980). *L. variegatus* are important members of freshwater food webs, both as consumers (Brinkhurst *et* al., 1972) and as a prey species (Zaranko *et* al., 1997; Wallace *et* al., 1998; Marsden and Bellamy, 2000). They form an important link in the trophic transfer of persistent environmental contaminants and feed mainly on the accumulated detritus of the benthic environment, e.g. algae, decaying organic matter, etc. (Brinkhurst and Jamieson, 1971). When feeding, *L. variegatus* use their head to forage in the sediment while the tail end, which is specialised for gaseous exchange, projects upwards towards the surface (Drewes, 2004). This facilitates gaseous exchange between the air/water and the pulsating blood vessel lying beneath the worm's epidermis (Drewes, 2004). Sediment is ingested, the digestible portion is assimilated and the undigested remaining sediment is excreted through the anus as faecal pellets (Appleby and Brinkhurst, 1971). This positioning and feeding strategy of the worms means that they will come into contact with contaminants both in the sediment and in the water column and so this is another benefit to using this species. Sediment species are essential to ecosystems for biogeochemical transformations, the maintenance of

clean water, the decomposition of organic matter, the transfer of materials and as a food sources for other species (Freckman *et al*., 1997) and so if the feeding behaviour of the worms is inhibited by a toxic substance it can have a profound effect on ecosystem functioning.

1.4.2 Reproduction in L. variegatus

The maximum body length of *L. variegatus* is approximately 10cm (approximately 200 – 500 segments) with a diameter of 1.5mm. Laboratory cultured worms tend to be smaller however and are usually about 4 - 6cm in length (Drewes and Brinkhurst, 1990). Wild specimens at 10cm are considered to be sexually mature hermaphrodites and although it has been rarely documented they can sexually reproduce by copulation and sperm exchange (Drewes and Brinkhurst, 1990). Sexually reproduced, transparent cocoons are produced with 4 – 11 fertilised eggs that undergo direct development. Worms hatch after approximately 14 days (Drewes and Brinkhurst, 1990). Reproduction under laboratory conditions is always asexual, however, and occurs via fragmentation and regeneration, i.e. transverse fission (Bely, 1999). Each fragment eventually grows into a normal sized worm which is made up of both new and old segments, representing two or more "generations" of development (Drewes, 2004). The advantages of this type of reproduction include survival after predatory attack and the ability to exploit resources as they become available (Christensen, 1984; Cook, 1969). Asexual reproduction and regeneration is found in several groups of annelid worms, including a number of oligochaete families (Brinkhurst and Jamieson, 1971). Because fragmentation occurs before any significant differentiation of head or tail segments the fission that occurs is termed architomy (Martinez et al., 2006). This form of asexual reproduction is rare in oligochaetes and paratomic fission is more common (Drewes and Fourtner, 1991). In paratomy the segments regenerate partially prior to fragmentation (Giese and Pearse, 1975). Reproduction by architomic fission must involve two processes. There must be a physical separation of an individual into two (or more) pieces and the reconstitution of a whole individual from each piece or at least two pieces (Bely, 1999). Each surviving fragment undergoes rapid regeneration to form a new head or tail (or both). The segments undergo morphallaxis where adult segments transform the fragment to match their new positional identity (Drewes and Fourtner,

1989). Each individual will eventually grow to a full size worm that represents two generations of development (Lesiuk & Drewes, 1999). As a result of this asexual reproduction under laboratory conditions, there is no life cycle stage of the organism within which it is possible to pick a universal time for testing. In order to ensure that all worms are in the same physiological condition worms are artificially fragmented twelve days prior to testing. Worms were cut approximately one third of the body length from the head using a scalpel and were allowed to regenerate a new head twelve days prior to testing. This process is described in detail in Chapter 2, Section 2.2.2.

Lumbriculus species are recognised by a number of characteristics e.g. green/dark coloured anterior end, helical swimming, body reversal behaviour (Drewes, 1999), however identification down to species level is difficult due to a lack of obvious sexual characteristics (Gustaffson *et al*., 2009). Currently there are fourteen known species of *Lumbriculus* species and originally *L. variegatus* was thought to be one species. However, a paper published by Gustaffson *et al*. (2009) discovered that there are two distinct clades within what was originally thought to be one species. They also suggested that there may be a third clade but this suggestion required further exploration. Clades I and II are found in Europe and North America, while the suspected clade III is found in North America and Japan. Gustafsson *et al*. (2009) suggested that distinct clades could react differently to exposure to contaminants and this could have important implications for using *L. variegatus* as a model species, however the authors also stated that further research was required before nomenclature and taxonomy should be changed. Genetic variation was not considered within this thesis. There is currently no information in the literature to suggest that genetic variation was considered in ecotoxicological testing in any other laboratory which used *L. variegatus*.

1.4.3 Use of Lumbriculus variegatus in ecotoxicological studies

L. variegatus were chosen as a test species as they have been widely used throughout the literature for the testing of potentially harmful substances as a bioassay organism since the early 1970s (e.g. Alekseev and Uspenskaya, 1974). Since then, *L. variegatus* have been the most widely used oligochaete to test the toxicity of a number of

substances, including metals (e.g. Schubauer- Berigan *et al*., 1993; Chapman *et al*., 1999), metal oxides (e.g. Stanley *et al*., 2010), various chemicals (e.g. ammonia: Schubauer-Berigan *et al*., 1995; PCB: Fisher *et al*., 1999; PAHs: Monson *et al*., 1995; chlorophenols: Nikkilä *et al*., 2003) and more recently NPs (e.g. Baun *et al*., 2008, Petersen *et al*., 2008, Stanley *et al*., 2010, Pakarinen *et al*., 2011). They possess a number of traits that make them a useful test species: 1) they are readily available in the UK and are easily cultured under laboratory conditions, 2) they have a known chemical exposure history, i.e. they have been used to test the toxicity of a wide range of substances, 3) they have adequate tissue for chemical analysis, 4) they have a high tolerance for a wide variety of exposure scenarios, 5) they are exposed via all important routes of concern, 6) they are suitable for both long- and short-term exposures (Brunson *et al*., 1998) and 7) for the reasons discussed above of their feeding and locomotive behaviours (Ding *et al*., 2001). There are not many other species that would fit all these criteria.

1.5 An overview of the methodologies used in this thesis

1.5.1 Characterisation of ZnO nanoparticles

Full and thorough physicochemical characterisation of NPs is essential to confirm the NPs you are working with, to relate the characterisation to impacts on organisms and to allow comparisons between different studies. Within this thesis the ZnO NPs were characterised at Heriot Watt University (HWU) and at the Facility for Environmental Nanoscience Analysis and Characterisation (FENAC) at the University of Birmingham. FENAC is a facility that aims to produce reliable analysis of NPs under realistic conditions to improve the understanding of the behaviour of NM in the environment. This service is offered through a competitive process and is funded by the Natural Environment Research Council (NERC). At HWU, dynamic light scattering (DLS) was used to characterise the ZnO NPs and bulk particles and at FENAC the techniques used included Brunauer, Emmett and Teller Method for surface area analysis (BET), X-Ray diffraction (XRD), Atomic Force Microscopy (AFM), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Scanning Transmission Electron Microscopy (STEM) with Energy Dispersive X-ray Spectroscopy (EDX), and

Dialysis with Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Details of the techniques used and their outcomes can be found in Chapter 2.

1.5.2 Medium used within this thesis

United States Environmental Protection Agency (USEPA) hard synthetic water was used throughout the studies within this thesis. The OECD 225 (2007) protocol (which was adapted for use in some of the studies within this thesis) states that the water chosen for toxicity testing must be of a quality that will allow *L. variegatus* to grow and reproduce for the duration of the acclimation period and test period without showing any abnormalities in appearance or behaviour (OECD 225, 2007). The EPA HW medium was used as it was already in use with *Daphnia magna* cultures within the laboratory and worms had been successfully cultured in this medium for a long period of time by another student within the Nanosafety Research Group, Jon Mullinger.

1.5.3 The addition of humic acid

There is little information regarding the impact of natural organic matter (NOM) on NP fate, behaviour, bioavailability and toxicity (Lee *et al.*, 2011; Lowry *et al.*, 2012). The bioavailability and toxicity of NPs in aquatic ecosystems is related to their dispersion stability, sedimentation of the NPs in the water column and the dissolution of ionic species (Gao *et al.*, 2012). Organic matter can alter the surface of the NP by coating it (Baalouska, 2009; Chappell *et al*, 2011) and this surface coating has been shown to increase the stability of the NPs in solution and decrease agglomeration rates (Lin and Xing, 2008; Chappell *et al.*, 2009). This can have a significant effect on the particle dispersion (Gao *et al.*, 2012). A change in dispersion can affect the NP fate and behaviour as well as their bioavailability and toxicity (Gao *et al*, 2012). Lee *et al.* (2011) suggested that the toxicity of NPs released to the environment may be overestimated without considering the impact of NOM, which can be varied by the medium parameters and the characteristics of the NOM itself. For this reason it was decided that all studies carried out within this thesis should also be conducted with or without the addition of 5mg/L Suwannee River humic acid (HA). Suwannee River HA was chosen as it is considered an international standard for HA. The quantity of 5mg/L

HA was decided upon based on the available literature (Baalouska, 2009; Chappell *et al*., 2011; Lee *et al*, 2011; Gao *et al*., 2012; Ma *et al.,* 2013) and previous studies conducted within our laboratory group by Jon Mullinger.

1.5.4 Endpoints used within this thesis

Behavioural endpoint

The locomotor and other behaviours of *L. variegatus* are significant indicators of the physical, chemical and biological properties of sediments and water bodies (Drewes, 1997). The behaviours of *L. variegatus* are highly stereotyped and so are very useful as a biomarker for toxicity. A number of papers have used this stereotyped behaviour of *L. variegatus.* Some authors have employed electro-physical techniques to test the conductivity of neural pathways (Rogge and Drewes, 1993; Lesiuk and Drewes, 2001; Sardo and Soares, 2010), while others have used touch-evoked response techniques (Lesiuk and Drewes, 2001; Ding *et al*., 2001; O'Gara *et al.,* 2004; O'Gara *et al*., 2006). For example, O'Gara *et al*. (2004) used tactile stimulation, delivered via a soft probe, in order to assess whether there was any reduction in the worms' ability to employ body reversal and helical swimming techniques after exposure to copper. Further information on the nervous system and behavioural response of worms is discussed in Chapter 3, section 3.1.

Oxidative stress endpoint: GSH assay

Oxidative stress occurs when there is an imbalance between oxidants and antioxidants in a cell (Sies 1985, 1986, 1991) and can potentially lead to damage. Due to the physicochemical characteristics of nanoparticles they are expected to have potential for toxicity via mechanisms such as oxidative stress. Within this thesis the content of the antioxidant glutathione (GSH) in the cells of worms exposed to ZnO NPs and bulk particles was investigated as an indicator of oxidative stress using various techniques. Further information on oxidative stress, the GSH assay and the outcomes of this study can be found in Chapter 4.

Uptake and depuration endpoints

Many environmental contaminants have the potential to accumulate through the aquatic food chain which can expose higher trophic levels to the contaminant, including humans (Dawson *et al.*, 2003; Krysanov *et al.*, 2010). Within this thesis the uptake and depuration of ZnO NPs and bulk particles was investigated using inductively coupled plasma atomic absorption spectrophotometry (ICP-AAS). Further details of the bioaccumulation of contaminants in aquatic species, the endpoint and the technique can be found in Chapter 6.

Histology

Very few papers have been published to date which have used histology to investigate the anatomy of *L. variegatus*. One of the most important aspects of histology is the immediate preservation of the structure of the tissue (Méndez-Vilas & Díaz, 2010). In this thesis a number of fixatives were investigated to determine which was best to use with *L. variegatus*. Further information on histological techniques and the outcome of this study can be found in Chapter 7.

1.6 Aims of this thesis

This research was conducted in order to attempt to fill a gap in the literature on the ecotoxicity of ZnO NPs. As mentioned previously, the investigation into the effects of nanomaterials on the aquatic environment is of utmost importance (Baun *et al.*, 2008) as the use of nanotechnology in industry is on the increase. No published studies have investigated the toxic effects of ZnO NPs on *L. variegatus*. The overall aim of this research was to assess the effects of ZnO NPs on the aquatic sediment dwelling worm, *L. variegatus*, via various toxicological tests. This aim was addressed by using water only, artificial sediment tests and biochemical endpoints following adapted OECD and other standard protocols.

1.6.1 Aims by chapter

Chapter 2 – To characterise ZnO NPs in sterile water and EPA HW medium using numerous characterisation techniques at Heriot Watt University and at FENAC, at the University of Birmingham.

Chapter 3 – To investigate the effects of ZnO NPs and bulk particles on the mortality and behaviour of *L. variegatus* in water only acute toxicity tests.

Chapter 4 – To investigate the effects of ZnO NPs and bulk particles on the oxidative stress reaction of *L. variegatus* in water only acute toxicity tests.

Chapter 5 – To investigate the effects of ZnO NPs and bulk particles on the reproduction, mortality and behaviour of *L. variegatus* in chronic artificial sediment toxicity tests.

Chapter 6 – To investigate whether *L. variegatus* are able to uptake, accumulate and depurate nano and bulk particles of ZnO.

Chapter 7 – To visualize, using histological processes, whether the ZnO particles are taken up by *L. variegatus* and whether any histological changes are evident in the images taken.

Chapter 2 Characterisation of engineered zinc oxide nanoparticles

2.1 Introduction

To ensure that the nanotechnology sector remains sustainable, thorough and comprehensive assessments of the potential environmental risks posed by NMs need to be carried out. The main reasons for carrying out physicochemical characterisation are to ensure that you are working with what you think you were working with, to relate the characterisation to impacts on organisms and to allow comparisons between different studies. The study of NM physicochemical properties is an important component of risk assessment since it will allow for the accurate assessment of fate and behaviour and thus exposure (Hassellöv *et al*., 2008). There is currently little data available about the fate and behaviour of engineered NPs (Hassellöv *et al*., 2008) and so further research in the areas of particle characterisation, and how this relates to behaviour of NMs in the environment, is necessary. In the environment, natural colloids or nanomaterials interact with each other and with other naturally occurring larger particles. The formation of aggregates and agglomerates in natural systems occurs in accordance with physical processes such as Brownian motion, fluid motion, colloidal science and gravity. Aggregation/agglomeration depends on particle size and charge and results in the removal of small particles from biological and ecological systems (O'Melia, 1980). To quantify the stability of a nanomaterial in the environment, its stability in suspension and its tendency to aggregate must be predicted (Mackay *et al*., 2006). In certain circumstances, particles in suspension aggregate and eventually settle out of the solution under the influence of gravity. A theory, named DVLO theory (named after the scientists who developed it – Derjaguin, Landau, Verwey and Overbeek) was developed in the 1940s which was designed to describe the stability of colloidal systems. This theory suggests that if the particles have a sufficiently high repulsive force the dispersion will resist aggregation and the system will be stable. However if the repulsive force is weak then flocculation or coagulation will occur (Malvern Technical Note, 2010).

There are currently numerous techniques available for the characterisation of NMs. A technique such as Dynamic Light Scattering (DLS) can be used to assess the

hydrodynamic diameter and stability of the nanoparticles in suspension, Scanning Transmission Electron Microscopy (STEM) and Transmission Electron Microscopy (TEM) can be used to assess the hydrodynamic diameter and shape of nanoparticles and techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Brunauer, Emmett and Teller Method for surface area analysis (BET), X-ray Diffraction (XRD) and Atomic Force Microscopy (AFM) can provide information on the dissolution, surface area, crystalline structure and surface characteristics of the particles, respectively. As part of any ecotoxicological study it is essential that not just one, but a number of physicochemical characterisation techniques are employed to gain a better understanding of the behaviour of the nanoparticles in relevant media. It is also important to take into account that the techniques employed will have limitations and data interpretation will be very specific to the technique used. For example, some sizing techniques are based on diameter measurements (of which there are several different specific types), while others report different size averages depending on what the instrument responds to, i.e. particle numbers, volume, mass or optical properties. Each method also has limitations with regards to maximum and minimum size and concentration ranges. Therefore, it is necessary to take into account that part of the NM size distribution may be "hidden" from the instrument/method (Hassellöv *et al.*, 2008). The toxicity and reactivity of NPs have been shown to be both shape and size dependent (Pal *et al.*, 2007; Madden and Hochella, 2005). Furthermore, the behaviour of the NMs can be influenced by interactions with organic matter (Hassellöv *et al.*, 2008) and so it is important to investigate the NPs under various environmentally relevant scenarios.

2.1.1 Characterisation at Heriot Watt University and at the University of Birmingham

The characterisation work for this study was carried out at both Heriot Watt University (HWU) in Edinburgh, Scotland, and at FENAC at the University of Birmingham, England. The DLS and zeta potential studies were carried out at HWU, with all other studies carried out by Dr. Bjorn Stolpe at FENAC.

2.1.2 Description of characterisation techniques

As mentioned previously there are a number of techniques currently available to characterise NPs. This section will describe the techniques that were used in the present study, both at HWU and at FENAC.

Dynamic Light Scattering:

DLS determines the hydrodynamic diameter of the NPs using the fluctuations in the scattered light which is diffused by the particles in a given suspension. The technique is based on the theory of Brownian motion of the particles (Cecere *et al*., 2003). In the DLS instrument the intensity is measured over short periods of time and then the software correlates the intensity at time t0 with the intensity at time t0 + δt (Hassellöv *et al*., 2008). The main advantages of DLS are that it is a quick method that is readily available with low running costs (Bootz *et al*., 2003). It also has a large material size range (3 to >1000nm) and sample perturbation (i.e. the sample is not disturbed through e.g. shaking during measurement) is kept to a minimum (Ledin *et al*., 1994). The disadvantages lie in the interpretation of the results. Large particles or aggregations/agglomerations of particles will have such a great influence on the reading that the smaller particles will be ignored. As a result of this, suspensions that induce high agglomeration or samples which have been contaminated, e.g. with dust, will not read correctly during hydrodynamic diameter size analysis (Hassellöv *et al*., 2008). A further disadvantage is that the DLS results are based on the assumption that the particles being examined are spherical in shape (Malvern Technical Note, 2010). This is not always the case in nanoparticles suspensions.

When examining the results obtained from DLS it is important to consider a number of parameters. The result will depend on the polydispersity index (PdI) of the sample. Malvern have set a PdI of between 0 and 1 when using the Malvern Zetasizer, with a PdI below 0.5 being acceptable. This is an estimate of the width of the distribution of particle size in a sample (Malvern Technical Note, 2010). The intercept value is also an important consideration when interpreting the results and this refers to the intercept of the correlation function of scattered light intensity and is set by Malvern to be between 0 and 1 when using the Malvern Zetasizer, with an intercept value of 0.85

– 0.95 being acceptable (Malvern Technical Note, 2010). Finally, Malvern state that the count rate (the number of particles in the sample) should be at least 10 kilo counts per second (kcps) above the dispersant count rate in order to ensure enough particles are in the suspension (Malvern Technical Note, 2010).

Zeta Potential:

According to Sze *et al*. (2003), when the surface of a NP comes into contact with a solution a charge forms between the surface of the particle and the aqueous solution. This disrupts the arrangement of the ions in the solution and produces a thin region of non-zero net charge density near the surface. The arrangement of charges at the surface and the ions in the solution is referred to as the electrical double layer (EDL). A thin layer of counter-ions lie next to the charged surface and this is known as the compact layer (Sze *et al*., 2003) or stern layer (Malvern Technical Note, 2010). Ions found outside the compact layer are known as the diffuse layer (Sze *et al*., 2003). The counter-ions in this compact layer are immobile due to the strong electrostatic attraction, however, the counter-ions outside the compact layer are mobile (Sze *et al*., 2003). Within the diffuse layer there is a boundary at which ions and particles form a stable unit. When a particle moves so too do the ions within the boundary. The zeta potential is the electrostatic potential at this boundary between the compact and diffuse layers (Malvern Technical Note, 2010). The magnitude of the zeta potential within a system gives an indication of the stability of the system. If all the particles in a solution have a large positive or negative zeta potential they will tend to repel each other, however, if the particles have a low (absolute value) zeta potential, there is no force to prevent the particles from forming aggregates. Generally if particles have a more positive zeta potential than +30mV or more negative zeta potential than -30mV then they are considered stable. However, if the particles have a density that differs from that of the dispersant, they will eventually sediment or form aggregates (Malvern Technical Note, 2010). There are a number of factors which can affect the zeta potential of particles in suspension, including pH, conductivity and the concentration of a formulation component, so zeta potential can only be described in conjunction with these factors (Malvern Technical Note, 2010).

Brunauer, Emmett and Teller Method for surface area analysis (BET):

The BET method is used extensively in surface science for calculating the surface area of a solid particle by the adsorption of gas molecules onto that particle. Brunauer, Emmett and Teller derived an equation that suitably interpreted multilayer gas adsorption isotherms and gave information as to the surface area of solid catalysts (Brunauer *et al*., 1938). BET surface areas have been found to be in agreement with calculations on values derived from electron micrographs (Raj, 2002).

X-ray Diffraction:

X-rays are high energy beams of electromagnetic radiation and when one of these beams meets an atom two processes may occur; 1) the beam may be absorbed with an ejection of electrons from the atoms or 2) the beam may be scattered (Warren, 1969). The interference of the beam scattering is referred to as diffraction which is the constructive interference of one or more scattered waves (Suryanarayana and Grant Norton, 1998). The method determines, via the scattering of x-rays, the arrangement of atoms within a crystal using the angle and intensities of the scattered beam. Scattering of x-ray beams is widely used for determining the atomic structure of a substance (Harding *et al*., 1987).

Atomic Force Microscopy (AFM):

AFM images show significant information about the surface characteristics of a sample with unprecedented clarity (Braga & Ricci, 2004). AFM is a tool which images the topography of a solid surface at high resolution. During AFM, the sample is scanned by a tip which is mounted on a cantilever spring. While moving, the force between the tip and the sample is measured based on the deflection of the cantilever. An image of the surface topography is obtained by plotting these deflections (Butt *et al*., 2005).

Transmission Electron Microscopy (TEM):

TEM makes use of electron properties, both as particles and as waves. A beam of electrons is transmitted through a very thin specimen of the sample of interest. These electrons interact with the sample as they pass through it and an image is produced from this interaction. The advantage of using TEM is that it has a very high resolution but it is limited in sample size – the higher the resolution of an instrument the less of the sample can be seen (Williams & Carter, 2009).

Scanning Electron Microscopy (SEM), Scanning Transmission Electron Microscopy (STEM) and Energy Dispersive X-ray Spectroscopy (EDX):

SEM and STEM are very powerful techniques that operate at the nanoscale. Secondary or backscattered electrons are used for imaging in SEM but in STEM higher signals and better special resolution are available by detecting transmitted electrons. Image formation is dependent on signals produced by the interactions between an electron beam as it scans the specimen and the specimen itself. The advantage of using SEM over TEM is that the samples do not have to be as thin as TEM, however the resolution of SEM is less than that of TEM. The advantage of using STEM is that it is suitable for use with further analysis techniques, such as energy dispersive x-ray spectroscopy (EDX) (Sawyer *et al*., 2008). EDX is a standard technique for element identification. It is attached to an SEM or STEM and uses the primary beam of the microscope to generate x-rays. The composition of the sample of interest is analysed based on the energy of these x-rays (Hollerith *et al*., 2004).

Dialysis and Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

Dialysis is a method by which ions are separated from NPs in media by allowing them to pass through dialysis bags made of regenerated cellulose. Solutes diffuse across a selectively permeable membrane down a concentration gradient in an effort to achieve equilibrium (http://www.nanocomposix.com, 05/09/2012). Once separated, the Zn concentration can be measured with ICP-MS. ICP-MS measures most elements on the

periodic table and is used to quantitatively measure elements in a sample (Perkin Elmer Technical Note, 2012).

2.1.3 Aim of DLS and zeta potential study

The aim of this study was to determine the level and rate of agglomeration of ZnO NPs and bulk particles in EPA HW medium and sterile water, both in the presence and absence of organic matter (Suwannee River Humic Acid, HA), over a time period of 0 to 96 hours, under experimentally relevant conditions. The null hypothesis in this experiment was that time or the addition of HA did not affect the level and rate of agglomeration in ZnO NPs and bulk particles in EPA HW medium. A further aim was to investigate the usefulness of this method of characterisation for use with complex medium and polydispersed suspensions of ZnO NPs. A second study was conducted to assess the hydrodynamic diameter and zeta potential of the ZnO NPs and bulk particles under "pristine" conditions, i.e. filtered medium suspensions and deionised water samples, at the recommended concentrations as opposed to experimental concentrations, at one time point immediately after sonication. Concentrations, as recommended by the Malvern user guide, were used, since concentrations used in ecotoxicology experiments were too low.

2.1.4 Aim of FENAC studies

The aim of the studies conducted at FENAC was to fully characterise the ZnO NP and bulk particles that were to be used in all toxicity testing. The characterisation was performed on the powder form of ZnO NP as well as suspensions in EPA HW medium with and without 5mg/L HA, depending on the technique. The null hypothesis examined in these studies was that the addition of HA would not affect the characteristics of the ZnO NPs when compared to those suspended in EPA HW medium alone.

2.2 Methods:

2.2.1 DLS study

Dispersion medium:

The EPA HW medium was made according to the method outlined in Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition, October 2002 (USEPA, 2002). The constituents of the medium can be seen in Table 2.1.

Table 2.1: Constituent salts of reconstituted hard water (USEPA, 2002).

Salt	**Concentration (mg/L) in DI water**
Calcium sulphate dihydrate ($CaSO_4.2H_20$)	120
Magnesium sulphate ($MgSO_4$)	120
Potassium chloride (KCl)	8
Sodium hydrogen carbonate ($NaHCO_3$)	192

The analytical reagent grade salts were sourced from Fisher Scientific, Loughborough, U.K. First, 2.4g $CaSO_4.2H_2O$ was dissolved in 4L deionised water in two 2L beakers overnight. The following day the solution was added to the water butt. Following this 2.4g $MgSO_4$ and 0.16g KCl were dissolved in 1.8L of deionised water in separate 2L beakers for 15-20 minutes. These were added to the water butt. Finally, 3.84g $NaHCO_3$ was dissolved in 1.8L of deionised water for 15-20 minutes and then washed into the butt. The water butt was then topped up to 20L. Aeration was added to the butt via an air pump attached to standard aquarium tubing for at least two hours, but preferably overnight. Sterile water was sourced from Baxter, U.K. Deionised water was sourced from HWU.

The ZnO NPs and bulk were sourced from Alfa Aesar, Germany. The ZnO NP product was named "Zinc Oxide Nanogard" with a lot number of E06T060 and the ZnO bulk product was named "Zinc Oxide ACS 99% min." with a lot number of E26331. Stock suspensions of nanoparticles were sonicated for 30 minutes at 20ºC. Suspensions of NPs and bulk zinc oxide at concentrations of 1.25, 2.5, 5 and 10mg/l, with and without 5mg/l humic acid, were made from stock suspensions. Suwannee River humic acid (HA) (sourced from the International Humic Substances Society, USA) was added to the stock suspensions as required after sonication. 5mg/L stock suspensions of HA

were prepared by adding 5mg HA to 1L of EPA HW medium and stirring (with a magnetic stirrer) for 15 minutes. The humic acid was added after sonication as sonication can cause the acid to change (Naddeo *et al*., 2007). Oxidation, pyrolysis or mechanical degradation of the humic acid molecules can occur in an ultrasonic field (Naddeo *et al*., 2007). Once the suspensions had been prepared from the stock they were immediately read on the Zetasizer for the 0 hour time point.

DLS (96 hour study):

The Malvern Zetasizer Nanoseries, Nano – ZS, was switched on at least 30 minutes prior to the beginning of the experiment each day to prevent thermal equilibration problems which can affect measurement results. Both hydrodynamic diameter and zeta potential readings were taken at 0, 24, 48, 72 and 96 hours. Samples were taken from the top 1-2cm of solutions at each time point using a 2ml syringe, with care not to disturb the solution to prevent the re-suspension of any settled particles.

A hydrodynamic diameter SOP was run for both ZnO NPs and ZnO bulk particles which contained the particle and medium parameters. Three readings were taken per sample and within these three readings the machine ran a number of times (amounts of runs determined by the machine based on the sample) to determine the hydrodynamic diameter. A zeta potential SOP was prepared for both ZnO nanoparticles and ZnO bulk particles which contained the particle and medium parameters. After the hydrodynamic diameter readings were taken the zeta potential SOP was opened and run in the same manner as described above i.e. three readings were taken per sample with numerous runs per reading.

DLS (size measurement study):

Solutions of NPs and bulk zinc oxide at concentrations of 0.1 and 0.01mg/ml, with and without 5mg/l humic acid, were made from the stock suspensions. Prior to making up the stock solutions, however, the medium and deionised water were filtered through a 0.2µm filter, with the first few drops discarded from each filter. Stock solutions of NPs were sonicated for 30 minutes at 20ºC. Suwannee River humic acid was added to the

stock solutions as required after sonication. As previously described the humic acid was added after sonication as sonication can cause the acid to disassociate (Naddeo *et al*., 2007). Once the suspensions had been prepared from the stock they were immediately read on the Zetasizer. The Malvern Zetasizer Nanoseries, Nano –ZS, was switched on at least 30 minutes prior to the beginning of the experiment each day to prevent thermal equilibration problems which can affect measurement results. Both hydrodynamic diameter and zeta potential readings were taken as previously described in the DLS 96 hour study.

Statistical analyses:

Statistics were carried out on these data using Minitab 15 Statistical Software. Details of the tests carried out are detailed in the appropriate sections. The p value was accepted at 0.05.

2.2.2 FENAC studies

These studies were carried out at FENAC at the University of Birmingham by Dr. Bjorn Stolpe. Work was conducted over a two week period in January 2012. I attended the facility during this time to learn about the techniques and gain relevant training, however the majority of data gathering, analysis and reporting was done and produced by Dr. Stolpe. Each section written by Dr. Stolpe has been referenced as "Report FENAC/2010/2012/003, Dr. Bjorn Stolpe". I conducted parts of the studies (e.g. exposure of the worms, preservation of worms for sectioning etc.) and this will be noted by unreferenced methods.

Culturing of test species

The cultures of *L. variegatus* were held in hard reconstituted water adapted from the US Environmental Protection Agency (USEPA) guidelines (2002). The constituents of the water can be seen in table 2.1. This medium was made according to the protocol described in section 2.2.1.

L. variegatus cultures were maintained according to a protocol that was adapted from the OECD Guidelines (2007). *L. variegatus* cultures were stored in 3 x 6L tanks in an incubator at 20 ± 1°C with a light regime of 16:8 hours. The substrate used within the tanks was cut strips of unbleached brown paper towels. The culture medium was renewed once per week by sieving the paper towels through a 500µm mesh into a large plastic tub. The culture tanks were then scrubbed in tap water and rinsed in culture medium. The paper towels were replenished if necessary and the worms were rinsed from the sieve, using culture medium, back into the clean tanks. Worms were also collected from the bottom of the plastic tub and returned to the tanks. The culture tank was then topped up with approximately 5L of culture medium. Following this 0.7g of pre-powdered aquaria tropical flake food (Tetramin, Tetra, Germany) was added to each tank. The tanks were replaced in the incubator with aeration (filtered air) provided via an air pump attached to standard aquarium tubing and glass pipettes.

Synchronisation and exposure of worms prior to characterisation analysis:

Twelve days prior to testing worms were artificially fragmented according to a protocol adapted from the OECD Guidelines 225 (2007). Large worms, that did not show signs of natural fragmentation, were chosen and transferred from the culture aquarium to a glass petri dish which contained a small amount of EPA HW medium. The worms were dissected along the median body region using a scalpel. Care was taken that the posterior ends were of similar size. Posterior parts of the worms were transferred to a 1L beaker containing EPA HW medium and shredded unbleached, prewashed and autoclaved paper towels. The beakers were then placed in an incubator (at 20°C ± 1°C) covered with parafilm and with aeration. At day 7 of regeneration one beaker of worms were fed 0.35g pre-powdered aquaria tropical flake food ("TetraMin", Tetra, Germany). After regeneration, worms that were actively swimming or crawling upon gentle stimulus were used in testing.

The ZnO NPs were weighed using Sartorius CP2245 electric scales onto anti static trays in a laminar flow hood. Once weighed the 0.02g of NPs were transferred to a volumetric flask containing 40ml EPA HW medium and were sonicated for 30 minutes. Suspensions of 1.25mg/L, 2.5mg/L, 5mg/L and 10mg/L ZnO NP were made up from the sonicated stock. When the nanoparticle solutions were prepared the

temperature, dissolved oxygen and pH were measured. In order for a test to proceed (according to the OECD Guideline 225, 2007) the parameters needed to be measured at certain times and be within the ranges specified in table 2.2. Although this test was not done following the OECD protocol exactly these parameters were still held as best practice for the adapted protocol.

Table 2.2: Parameters for testing (OECD Guideline 225, 2007).

Parameter	**Level**	**Checked**
Temperature	20°C ± 2°C	1 replicate at the beginning and end of testing
Dissolved oxygen	> 30% saturation	1 replicate at the beginning and end of testing
pH	Between 6.0 – 9.0	1 replicate at the beginning and end of testing

The dissolved oxygen was measured with a Hach LDO HQ10 O_2 dissolved oxygen meter. The temperature was also measured using the DO meter which was inserted into the medium and gently stirred for 30 seconds or so until the temperature stabilised. The probe was allowed to run until the percent oxygen saturation stabilised. The pH was measured using a pHM210 standard pH meter (MeterLab Radiometer Analytical). The probe was inserted into the medium and gently stirred until the pH stabilised. After determination of each solution's parameters 20ml of the required solution was added via a 20ml syringe to each glass vial. After the solution was added one worm was added per vial. The vials were then moved to the 20 ± 2°C controlled temperature room and arranged at random under a light regime of 16:8 hour light:dark. They were left in the controlled temperature incubator for 96 hours. During the 96 hour period vials were observed for any obvious changes in behaviour when compared to the controls or mortalities. Any observations were recorded. After 96 hours the worms were removed and preserved for testing as outlined below.

<u>BET:</u>

The total surface area of ZnO NPs and bulk particles was assessed using the BET gas adsorption method. Amounts of 0.1 – 0.3g of ZnO NPs and bulk particles were outgassed under vacuum at 200˚C in BET-vials during 12 hours. Following this the

samples were weighed precisely (± 1 mg precision) and analysed using a SA 3100 Surface Area Analyzer (Beckman Coulter). The temperature was controlled by immersing the vials in liquid nitrogen. The gas was used to determine the free space in the vials and adsorption isotherm of N_2 gas to determine the total surface area of the sample (0.05-0.2 relative pressure range). The correlation coefficients of the adsorption isotherms were always > 0.999. The BET surface area was calculated by dividing the total surface area by the sample mass (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

XRD:

Approximately 0.1 g of dry powdered ZnO-particles were analysed using XRD (Bruker D8 Advanced X-ray diffractometer, 5˚ to 90˚ 2θ, 0.0197˚ step size, 0.38 s count time). The XRD spectra were background corrected and the peak width at half peak height was measured at the characteristic peaks for ZnO at 2θ = 32.7˚, 34.4˚, 36.2˚, 47.5˚, 56.6˚, 62.9˚ and 68.0˚. The instrument peak broadening was measured by determining the variation in peak width at half peak height with 2θ on a reference Al_2O_3 sample (NIST). The peak broadening caused by nanometre-sized ZnO-particles (β) was thereafter calculated by subtracting the instrument broadening from the peak widths at half peak height of the ZnO-particles. The crystallite diameter (τ) was calculated using the Scherer equation (equation 1), where k = shape factor (0.95) and λ = X-ray wavelength (1.54 Å).

$$\tau(\hat{A}) = \frac{k\lambda}{\beta cos\theta} \qquad (1)$$ (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Sample preparation for AFM and TEM:

Dispersions of 0.1 mg/L and 1 mg/L ZnO NPs were prepared in milli-Q water, EPA HW medium and EPA HW medium with 5mg/L HA. Freshly cleaved sheets of muscovite (around 50 × 50 mm) and 300 mesh Formvar/carbon coated Cu TEM grids were placed in flat bottomed ultracentrifuge tubes, 10 mL of the ZnO-particle suspensions were added to the tubes, and the NPs were deposited onto the muscovite

and TEM-grids by 30000 rpm ultracentrifugation (Beckman L-75) during 60 min. The muscovite and TEM-grids were cleaned from salt and un-adsorbed particles by gentle immersion in milli-Q water, and were thereafter dried under cover overnight. The same method was used to prepare samples after worm exposures, but 2.5mg/L and 10mg/L ZnO-suspensions were used and 300 mesh holey carbon coated Cu grids were used. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Atomic Force Microscopy (AFM):

Sizes and shapes of the nanoparticles were analysed by AFM using a XE100 instrument (Park Systems). The samples were analyzed in non-contact mode using a Si cantilever (PPP-NCHR, Park Systems), having a frequency of 300 Hz and a force constant of 42 N m^{-1}. Images of 2×2 µm, 5×5 µm and 30×30 µm were acquired and particle size distributions were determined by measuring the heights of at least 165 particles in each sample, using the XEI software (Park Systems). (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Transmission Electron Microscopy (TEM):

Micrographs of the raw ZnO-particles and particles in the worm exposure media were acquired at 30k, 100k, 300k and 500k magnification using a TEM instrument (JEOL 1200EX) with LaB_6 filament, operating at 80 keV. The micrographs were analysed using specially designed computer software (Digital Micrograph, (Gatan Inc.). On most of the images, the particles were part of larger aggregates, and two different approaches were used for size and shape analysis. In the first approach, individual nanoparticles were manually selected by marking their borders on the TEM-images, and their dimensions including length (l), breadth (b), equivalent circular diameter (d), area (A), convex area (A_c), perimeter (P) and convex perimeter (P_c) of each particle were given by the software. From this information, the 'form factor' and 'roundness', describing the degree by which the 2-dimensional shape of the particles deviated from circular shape, were calculated using equations 2 and 3:

$$formfactor = \frac{4\pi \times A}{P_e^2} \qquad (2)$$

$$roundness = \frac{4A}{\pi \times L^2} \qquad (3)$$

In the second approach, whole aggregates of particles were automatically selected by the software and their dimensions were measured. In addition to the form factor and roundness, the 'convexity' and 'solidity' of each aggregate, describing how 'convex' or 'branched' the aggregates were, was calculated using equations 4 and 5:

$$convexity = \frac{P_c}{P} \qquad (4)$$

$$solidity = \frac{A}{A_c} \qquad (5)$$ (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Preparation of Tissue Samples for analysis with STEM-EDX:

Worms exposed to ZnO-particles were fixed in gluteraldehyde (1 hour) followed by fixing in osmium tetroxide (1 hour) and dehydration in alcohol/water mixture with successively increasing concentration of alcohol (70, 90 and 100 %, each 15 min). The fixed worms were immersed in a 1:1 mixture of a resin (consisting of Araldite CY212, AGAR 100 resin, DDSA, DBP and DMP30) and propylene oxide during 45 min and were thereafter embedded in the mixture in a vacuum oven at 500-600˚C for 20 minutes. The resin was thereafter polymerized 60˚C and atmospheric pressure for 16 hours. 100-120 nm sections of the embedded samples were cut using a Reichert-Jung Ultracut E ultramicrotome, and the sections were deposited on 200 mesh Formvar/carbon coated Cu grids. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Scanning transmission electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDX):

A scanning electron microscope (JEOL 7000F) with STEM and EDX units (Oxford Inca) were used to assess the uptake of ZnO-particles into worms. Micrographs of the sectioned worm tissue samples were acquired with a 20 keV electron beam in both STEM mode and in SEM mode with backscattered electron detection. Various areas of the tissue was analysed, but emphasis was laid on the gut of the worm, in order to

investigate the uptake of whole ZnO-particles. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe)

Dialysis:

The production of soluble Zn (< 1 kDa species e.g., Zn^{2+}) from dissolution of ZnO-particles was assessed using dialysis. ZnO-particle suspensions (10 mg/L, 2L each) were prepared in EPA HW medium and EPA HW medium with humic acid. Dialysis bags of regenerated cellulose (SnakeSkin, 1 kDa Molecular Weight Cut Off (MWCO)) were filled with 100 mL of medium without ZnO-particles, were closed with specially designed clips (SnakeSkin, 50 mm) and immersed in the suspensions with ZnO-particles. Two replicate bags were used for each ZnO-particle suspension. 5 mL samples were withdrawn from the bags at 17 occasions over a period of 33 days and were immediately acidified by adding 100 µL of concentrated ultrapure HNO_3 to each 5 mL sample. The samples were diluted 100 times with milli-Q water, and again acidified to 1 % with ultrapure HNO_3, before analysis of Zn-concentrations using ICP-MS (Agilent 7500ce with collision cell) (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

2.3 Results

2.3.1 DLS and zeta potential study

Due to the large data output of this study graphical representation of these data can be found in Appendix A.

Upon investigating the parameters within the NP study, 54% of the PdIs were greater than 0.5, 6% "met the quality criteria" according to the export reports, 47% of the readings did not have 10kcps in excess of the media and 78% of the data did not have an intercept between 0.85 and 0.95 and in the zeta potential study 6% of the data "met the quality criteria", indicating that the results obtained for a large proportion of the samples tested were not reliable. Upon investigating the data in the bulk particle study 55% of the PdIs were greater than 0.5, 6% "met the quality criteria" according to the export reports, 43% of the readings did not have 10kcps in excess of the media, 96% of the data did not have an intercept between 0.85 and 0.95 and within the zeta potential

study 10% of the data "met the quality criteria", again indicating that that the results obtained for a large proportion of the samples tested were not reliable.

Hydrodynamic Data (HD)

Table 2.3 below shows the experimental design for the 96 hour HD DLS study. The study investigated 4 concentrations (1.25, 2.5, 5 and 10mg/L), 5 time points (0, 24, 48, 72 and 96 hours), 4 media types (EPA HW and DI water with and without HA) and 2 particle types (NP and bulk).

Table 2.3: The experimental design for the 96 hour HD DLS study.

Experiment	**Test**	**Factors**	**Interaction Factors**
DLS HD 96 hour	GLM	Concentration (4)	Conc*Time
			Conc*Media
			Conc*Particle
		Time (5)	Time*Media
			Time*Particle
			Media*Particle
		Media type (4)	Conc*Time*Media
			Conc*Time*Particle
		Particle type (2)	Conc*Media*Particle
			Time*Media*Particle

Statistics were carried out on these data using Minitab 15 Statistical Software. P was set at 0.05 for all studies. A Kolmogorov Smirnov test was performed to check for normality. Data were found to be non compliant with requirements of parametric tests and so were transformed via log transformation. After transformation data were found to be compliant and a general linear model (GLM) was performed to analyse the data. The output from the GLM can be seen in Table 2.4.

Table 2.4: GLM output table for hydrodynamic diameter 96 hour study examining 4 factors (concentration, time, media type and particle type) and the interaction factors between these factors.

	Source	DF	Seq SS	Adj SS	Adj MS	F	P
Factors	Concentration	3	0.95690	0.98309	0.32770	3.89	0.017
	Particle	1	0.82611	0.84089	0.84089	9.98	0.003
	Media	3	28.85406	27.11036	9.03679	107.25	0.000
	Time	4	23.48006	22.92339	5.73085	68.01	0.000
Interactions	Conc*Particle	3	0.36599	0.34273	0.11424	1.36	0.272
	Conc*Media	9	1.00668	0.97817	0.10869	1.29	0.277
	Conc*Time	12	2.49315	2.43667	0.20306	2.41	0.021
	Particle*Media	3	1.47594	1.41719	0.47240	5.61	0.003
	Particle*Time	4	4.75169	4.64227	1.16057	13.77	0.000
	Media*Time	12	2.22929	2.23902	0.18659	2.21	0.033
	Conc*Particle*Media	9	1.49774	1.45579	0.16175	1.92	0.081
	Conc*Particle*Time	12	1.20982	1.08007	0.09001	1.07	0.414
	Conc*Media*Time	36	3.21542	3.22132	0.08948	1.06	0.430
	Particle*Media*Time	12	2.30776	2.30776	0.19231	2.28	0.029
	Error	35	2.94910	2.94910	0.08426		
	Total	158	77.61971				

The concentration of ZnO ($P = 0.017$), the time point ($P < 0.001$), the media type ($P < 0.001$) and the particle type ($P = 0.03$) were all found to be factors in determining size differences. Tukey tests were performed in order to ascertain where these differences lay. Within the concentration factor only the control was found to be significantly different ($P = 0.037$) from 10mg/L ZnO. Within the time factor time 0 was significantly different from times 24, 48, 72 and 96 ($P < 0.001$) and time 24 was significantly different from 72 hours ($P < 0.001$) and 96 hours ($P < 0.001$). Within the media factor EPA HW media and EPA HW media with HA was significantly different from deionised water and deionised water with HA ($P < 0.001$). Interactions factors were also tested for significance in this model. It was found that the interactions between concentration and time ($P = 0.021$), particle and time ($P < 0.001$), particle and media ($P = 0.003$), media and time ($P = 0.033$) and between the particle, media and time ($P = 0.029$) were all found to be significant. This indicates that the effects of the factors were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to another factor (Dytham, 2011).

Zeta potential Data (ZP):

Table 2.5 below shows the experimental design for the 96 hour ZP DLS study. The study investigated 4 concentrations (1.25, 2.5, 5 and 10mg/L), 5 time points (0, 24, 48, 72 and 96 hours), 4 media types (EPA HW and DI water with and without HA) and 2 particle types (NP and bulk).

Table 2.5: The experimental design for the 96 hour ZP DLS study.

Experiment	**Test**	**Factors**	**Interaction Factors**
DLS ZP 96 hour	GLM	Concentration (4)	Conc*Time
			Conc*Media
		Time (5)	Conc*Particle
			Time*Media
			Time*Particle
		Media type (4)	Media*Particle
			Conc*Time*Media
			Conc*Time*Particle
		Particle type (2)	Conc*Media*Particle
			Time*Media*Particle

A Kolmogorov Smirnov test was performed to check for normality. Data were found to be compliant with requirements of parametric tests and a global general linear model (GLM) was performed to analyse the data. The output from the GLM can be seen in Table 2.6.

Table 2.6: GLM output table for 96 hour zeta potential study examining 4 factors (concentration, time, media type and particle type) and the interaction factors between these factors.

	Source	DF	Seq SS	Adj SS	Adj MS	F	P
Factors	Concentration	3	328.88	392.26	130.75	2.83	0.049
	Particle	1	234.33	265.64	265.64	5.75	0.021
	Media	3	1475.51	1660.46	553.49	11.98	0.000
	Time	4	1959.57	2069.87	517.47	11.20	0.000
Interactions	Conc*Particle	3	27.62	25.06	8.35	0.18	0.909
	Conc*Media	9	297.59	403.14	44.79	0.97	0.477
	Conc*Time	12	1003.85	1069.54	89.13	1.93	0.055
	Particle*Media	3	112.76	116.08	38.69	0.84	0.480
	Particle*Time	4	836.58	839.60	209.90	4.54	0.004
	Media*Time	12	1035.75	1041.32	86.78	1.88	0.063
	Conc*Particle*Media	9	555.46	568.08	63.12	1.37	0.231
	Conc*Particle*Time	12	432.19	381.28	31.77	0.69	0.754
	Conc*Media*Time	36	1002.01	1002.01	27.83	0.60	0.941
	Particle*Media*Time	12	1183.76	1151.42	95.95	3.35	0.003
	Error	34	2124.68	2124.68	46.19		
	Total	157	11426.78				

The concentration of ZnO ($P = 0.049$), the time point ($P < 0.001$), the media type ($P < 0.001$) and the particle type ($P = 0.021$) were all found to be significantly different from each other. Tukey tests were performed in order to ascertain where these differences lay. Within the concentration factor the control was significantly different from 1.25, 2.5 and 5mg/L ($P < 0.01$), 1.25, 2.5 and 5mg/L was significantly different from 10mg/L ($P = 0.034$). Within the time factor time 0 was significantly different from all other time points ($P = 0.023$). Time 24 was significantly different from 48 and 72 hours ($P = 0.04$) and 72 hours was significantly different from 96 hours ($P = 0.03$). Within the media factor EPA HW media was significantly different from deionised water with HA ($P < 0.001$), EPA HW medium with HA was significantly different from EPA HW medium ($P = 0.004$) and deionised water ($P < 0.01$) and deionised water was significantly different from deionised water with HA ($P < 0.001$). Interactions factors were also tested for significance in this model. It was found that the interaction between particle and time ($P < 0.001$) was found to be significant. This indicates that the effects of the factors were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to another factor (Dytham, 2011).

Summary of NP ZnO results:

Graphical representations of these data can be found in Appendix A. At 0 hours (Figure 1, Appendix A) the most stable suspension was sterile water with HA, all other suspensions were unstable. The largest hydrodynamic diameter was seen in the EPA HW with HA and sterile water suspensions at 10mg/L. At 24 hours (Figure 2, Appendix A) a high level of agglomeration was seen in the EPA HW suspension across all concentrations and this was reflected in the zeta potential results which were all greater than -30mV (i.e. between 0 and -30mV). At 48 hours (Figure 3, Appendix A) the EPA HW suspension had the highest level of agglomeration. At 72 hours (Figure 4) the EPA HW suspensions were all unstable. At 96 hours (Figure 5) no suspension appeared different from the previous time point of 72 hours.

Summary of Bulk ZnO Results:

At 0 hours (Figure 6, Appendix A) the EPA HW suspension had a hydrodynamic diameter that was larger than that of the sterile water and sterile water with H.A. suspensions. The zeta potential of the sterile with HA water suspensions indicate that they were stable since they were less than -40mV. At 24 hours (Figure 7, Appendix A) sterile water with HA were seen to be the most stable across the concentrations (zeta potential less than -30mV). At 48 hours (Figure 8, Appendix A) all suspensions were unstable. At 72 hours (Figure 9, Appendix A) EPA HW with HA suspension was stable at 10mg/L. The sterile water suspensions were stable at 72 hours at 1.25. 2.5 and 5mg/L. At 96 hours (Figure 10, Appendix A) only 5mg/L was seen to be stable. The sterile water with HA suspensions were stable at 2.5 and 5mg/L.

2.3.2 Size Measurement Results

Table 2.7 below shows the experimental design for the size measurement HD DLS study. The study investigated 2 concentrations (0.01 and 0.1mg/L), 2 HA (with and without HA), 2 media types (EPA HW and DI water) and 2 particle types (NP and bulk).

Table 2.7: The experimental design for the size measurement HD DLS study.

Experiment	**Test**	**Factors**	**Interaction Factors**
DLS HD Size Measurement	GLM	Concentration (2)	Conc*Time
			Conc*Media
			Conc*Particle
		HA (2)	Time*Media
			Time*Particle
			Media*Particle
		Media type (2)	Conc*Time*Media
			Conc*Time*Particle
		Particle type (2)	Conc*Media*Particle
			Time*Media*Particle

Figure 2.1 and 2.2 show the hydrodynamic diameter and zeta potential of ZnO NPs and bulk particles in filtered EPA HW medium and filtered deionised water, both in the presence and absence of 5mg/L humic acid. Even though the media were filtered some particulate matter was still picked up in the sample. Statistics were performed in order to assess if there were any significant differences between the different conditions.

<u>Hydrodynamic diameter data</u>

A Kolmogorov Smirnov test was performed to check for normality. Data were found to comply with parametric test requirements and a global general linear model (GLM) was performed to analyse the data. The output from the GLM can be seen in Table 2.8. The media type ($P = 0.011$) and the particle type ($P = 0.008$) were found to lead to significantly different results. Within the media factor, the EPA HW samples were found to have significantly higher HD than DI water samples. Within the particle factor, the bulk HD was significantly greater than NP HD. Interactions factors were also tested for significance in this model. It was found that the interaction between

concentration, particle and media (P = 0.048) was found to be significant. This indicates that the effects of the factors were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to another factor (Dytham, 2011).

Table 2.8: GLM output analysis carried out on hydrodynamic diameter data from the size measurement study examining 4 factors (concentration, time, media type and particle type) and the interaction factors between these factors.

	Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Factors	Concentration	1	85673	85673	85673	146.29	0.053
	Particle	1	4008004	4008004	4008004	6843.80	0.008
	Media	1	1797745	1797745	1797745	3069.71	0.011
	Addition of HA	1	29070	29070	29070	49.64	0.090
Interactions	Conc*Particle	1	3387	3387	3387	5.78	0.251
	Conc*Media	1	256	256	256	0.44	0.628
	Conc*HA	1	33343	33343	33343	56.93	0.084
	Particle*Media	1	21404	21404	21404	36.55	0.104
	Particle*HA	1	10609	10609	10609	18.12	0.147
	Media*HA	1	16307	16078	16078	27.45	0.120
	Conc*Particle*Media	1	103362	103362	103362	176.49	0.048
	Conc*Particle*HA	1	620	620	620	1.06	0.491
	Conc*Media*HA	1	16307	16307	16307	27.85	0.119
	Particle*Media*HA	1	106080	106080	106080	181.14	0.047
	Error	1	586	586	586		
	Total	15	6232525				

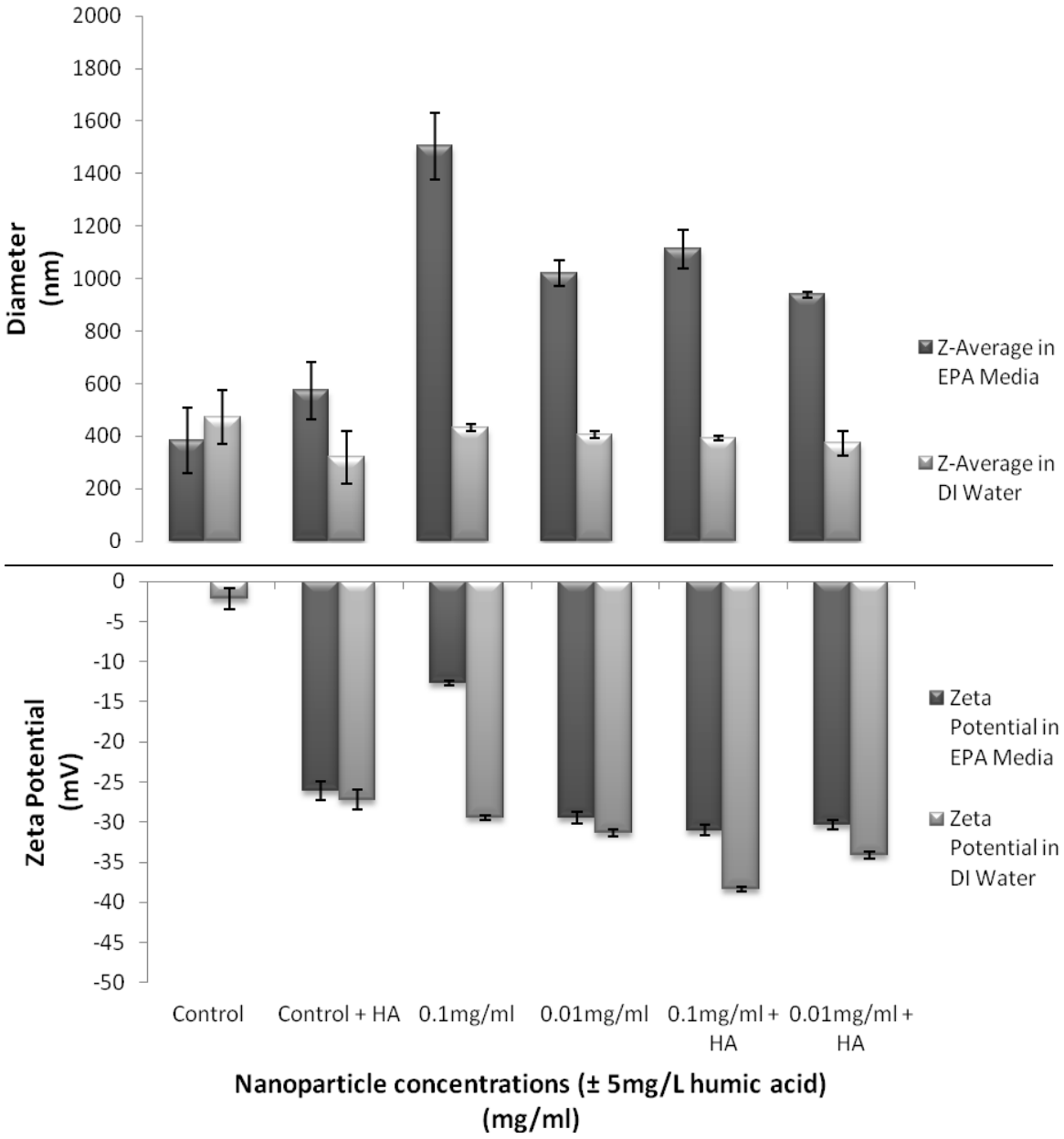

Figure 2.1: The hydrodynamic diameter and zeta potential of ZnO nanoparticles in filtered EPA HW media and filtered deionised water, both in the presence and absence of 5mg/L Suwannee River Humic Acid (Data represents means ± SE).

Zeta potential Data:

Table 2.9 below shows the experimental design for the size measurement HD DLS study. The study investigated 2 concentrations (0.01 and 0.1mg/L), 2 HA (with and without HA), 2 media types (EPA HW and DI water) and 2 particle types (NP and bulk).

Table 2.9: The experimental design for the size measurement ZP DLS study.

Experiment	**Test**	**Factors**	**Interaction Factors**
DLS ZP Size Measurement	GLM	Concentration (2)	Conc*Time
			Conc*Media
			Conc*Particle
		HA (2)	Time*Media
			Time*Particle
			Media*Particle
		Media type (2)	Conc*Time*Media
			Conc*Time*Particle
		Particle type (2)	Conc*Media*Particle
			Time*Media*Particle

A Kolmogorov Smirnov test was performed to check for normality. Data were found to comply with parametric test requirements and a global general linear model (GLM) was performed to analyse the data. The output from the GLM can be seen in Table 2.10. No factor or interaction was found to be significantly different.

Table 2.10: GLM output table for the size measurement zeta potential study examining 4 factors (concentration, time, media type and particle type) and the interaction factors between these factors.

	Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Factors	Concentration	1	166.80	166.80	166.80	4.46	0.282
	Particle	1	46.04	46.04	46.04	1.23	0.467
	Media	1	116.32	116.32	116.32	3.11	0.328
	Addition of HA	1	534.30	534.30	534.30	14.29	0.165
Interactions	Conc*Particle	1	36.18	36.18	36.18	0.97	0.505
	Conc*Media	1	3.55	3.55	3.55	0.10	0.810
	Conc*HA	1	361.57	361.57	361.57	9.67	0.198
	Particle*Media	1	16.12	16.12	16.12	0.43	0.630
	Particle*HA	1	59.52	59.52	59.52	1.59	0.427
	Media*HA	1	13.07	13.07	13.07	0.35	0.660
	Conc*Particle*Media	1	53.51	53.51	53.51	1.43	0.443
	Conc*Particle*HA	1	52.06	52.06	52.06	1.39	0.448
	Conc*Media*HA	1	0.17	0.17	0.17	0.00	0.957
	Particle*Media*HA	1	53.51	53.51	53.51	1.43	0.443
	Error	1	37.39	37.39	37.39		
	Total	15	1550.11				

Upon investigating the parameters within the NP study, 10% of the PdIs were greater than 0.5, 10% “met the quality criteria” according to the export reports, 66% of the readings did not have 10kcps in excess of the media and 40% of the data did not have an intercept between 0.85 and 0.95. In the ZP study 50% of the data “met the quality criteria”. Upon investigationg the data in the bulk particle study 90% of the PdIs were greater than 0.5, 0% “met the quality criteria” according to the export reports, 66% of the readings did not have 10kcps in excess of the media, 90% of the data did not have an intercept between 0.85 and 0.95 and within the ZP study 10% of the data “met the quality criteria”. These results indicated that not all of the results obtained for the samples tested were reliable.

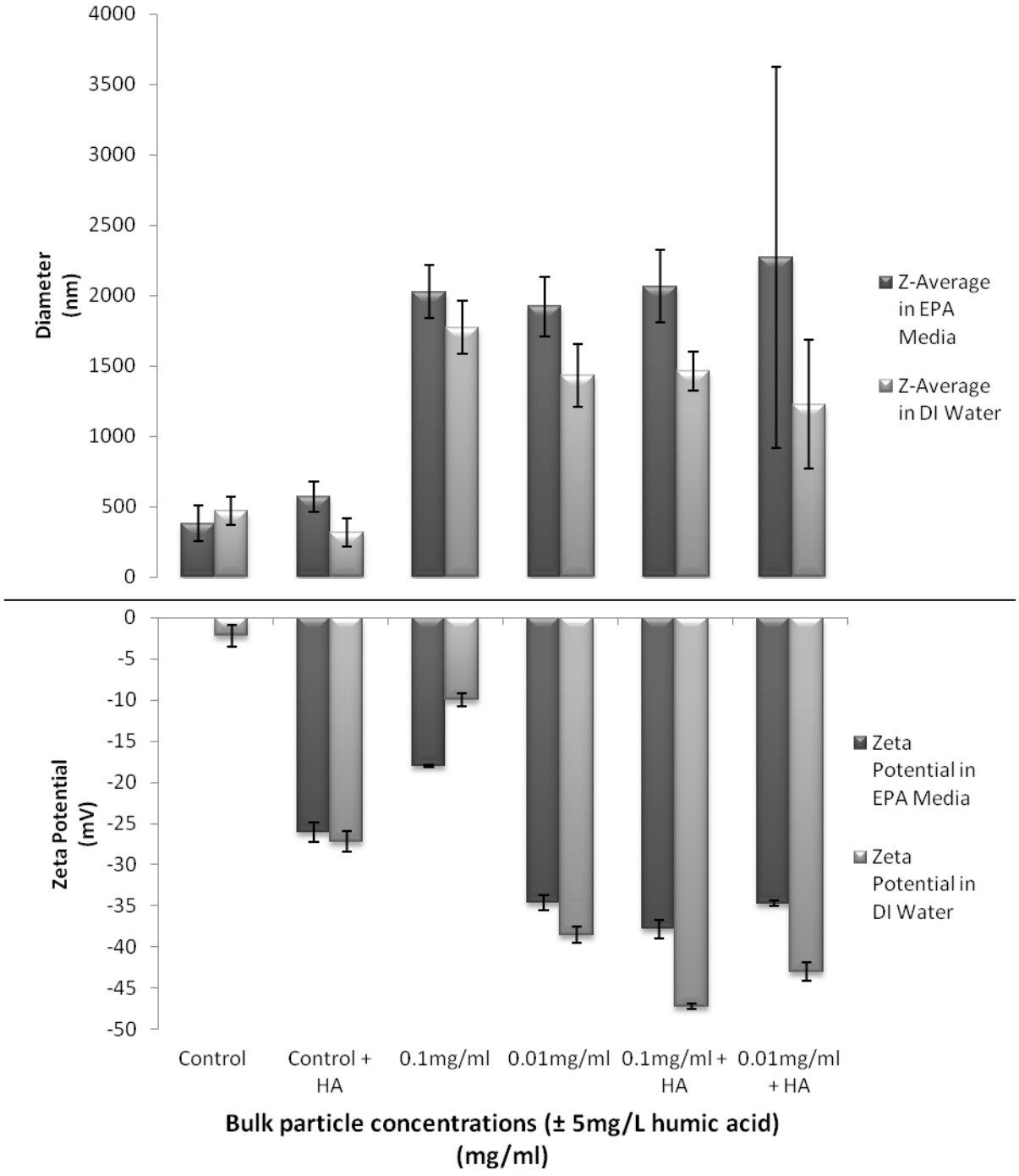

Figure 2.2: The hydrodynamic diameter and zeta potential of ZnO bulk particles in filtered EPA HW media and filtered deionised water, both in the presence and absence of 5mg/L Suwannee River Humic Acid (Data represents means ± SE).

2.3.3 FENAC studies

The following results were written by Dr. Bjorn Stolpe, FENAC, 2012 and each section is referenced as "Report FENAC/2010/2012/003, Dr. Bjorn Stolpe".

BET surface area

The BET surface area was 12.06 ± 0.023 m^2/g for ZnO NPs, and 5.8 ± 0.37 m^2/g for the ZnO-bulk particles. The average values and standard deviations are based on 3 replicate measurements each. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

XRD

The XRD-spectra of the ZnO nanoparticles and bulk particles were compared with a reference library and were confirmed to be particles composed of ZnO. The crystallite diameter (average value of the crystallite diameters determined at 2θ = 32.7˚, 34.4˚, 36.2˚, 47.5˚, 56.6˚, 62.9˚ and 68.0˚) were for nanoparticles 199 ± 53 nm and for bulk particles 208 ± 28 nm. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

TEM

The TEM-micrographs of ZnO suspensions in EPA HW medium showed particles that were several hundred nm in diameter, appearing to be composed of smaller nanoparticles aggregated or 'fused' together (Figure 2.3A, C; Table 2.11). The form factor and roundness (see below) of the aggregates of both nano and bulk particles in EPA HW medium were low (average form factor around 0.2, average roundness around 0.4-0.5) showing that the 2-dimensional shapes of the aggregates were far from circular (circular shape would give form factor = 1 and roundness = 1). The convexity and solidity of the aggregates of both nano and bulk-particles were also rather low (average convexity around 0.6, average solidity around 0.7), showing that the aggregates were highly 'branched' and concave (totally convex and solid particles would give convexity = 1 and solidity = 1). The TEM-micrographs from samples of EPA HW medium with humic acid showed particles with varying structure and morphology (Figure 2.3 B, D),

that looked rather different from the particles in EPA HW medium without humic acid. However, no significant differences in size, form factor, roundness, convexity or solidity could be shown between aggregates in EPA HW medium with and without HA (based on two-tailed T-test with 5 % significance). The numbers of aggregates used for the T-tests (6-42) were probably too low for the tests to be meaningful. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

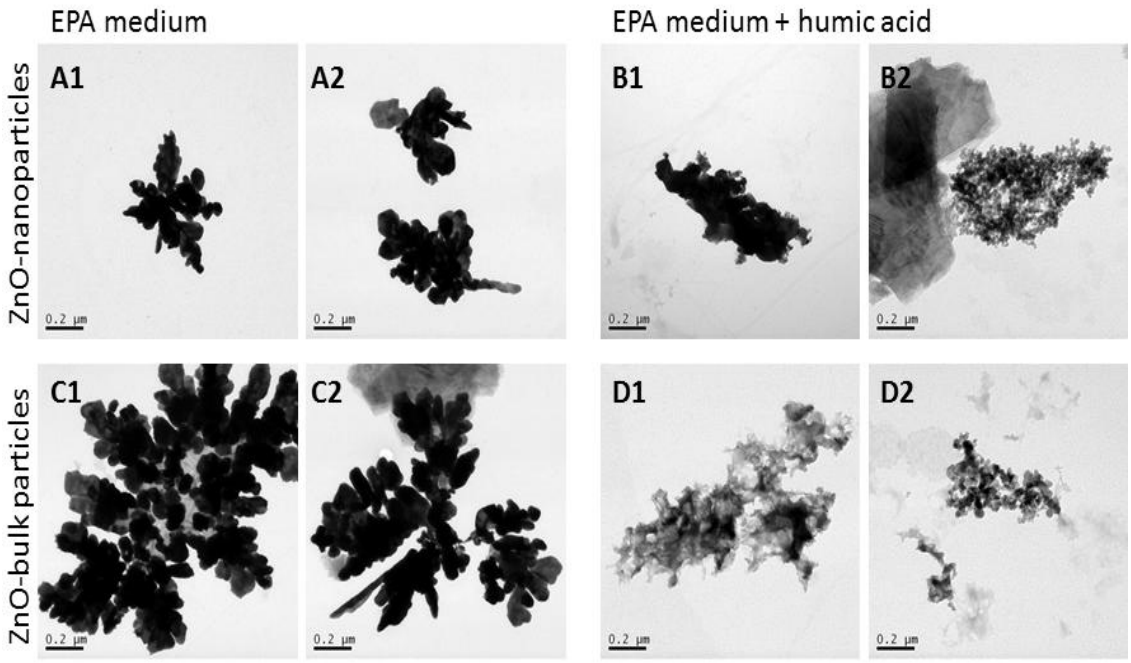

Fig. 2.3: TEM micrographs of ZnO-nanoparticles (A and B) and ZnO-bulk particles (C and D) in EPA HW medium (A and C) and EPA HW medium with humic acid (B and D).

Table 2.11: Size and shape factors of particle-aggregates in EPA HW medium with and without humic acid (mean ± SD).

	ZnO-nanoparticles		**ZnO-bulk particles**	
	EPA HW medium	**EPA HW with humic acid**	**EPA HW medium**	**EPA HW with humic acid**
Length (nm)	850 ± 380	670 ± 960	840 ± 460	890 ± 1000
Breadth (nm)	550 ± 210	360 ± 470	610 ± 330	550 ± 610
Equivalent circular diameter (nm)	720 ± 300	530 ± 740	730 ± 390	730 ± 840
Form factor	0.18 ± 0.075	0.2 ± 0.13	0.18 ± 0.088	0.2 ± 0.16
Roundness	0.4 ± 0.13	0.4 ± 0.13	0.47 ± 0.095	0.4 ± 0.12
Convexity	0.58 ± 0.087	0.6 ± 0.10	0.6 ± 0.10	0.6 ± 0.17
Solidity	0.68 ± 0.088	0.8 ± 0.51	0.71 ± 0.076	0.7 ± 0.11

The TEM micrographs from the EPA HW medium (where worms had been living during 10 days without ZnO-particles) showed large aggregates of small elongated (up to about 50 nm in length, 10 nm in breadth) particles (Figure 2.4A). It is possible that those particles were produced by the worms, or that they were formed by precipitation from the salts composing the EPA HW medium. Some micrographs (e.g., 2.4A1a) showed a diffuse 'matrix' surrounding the particles, probably organic matter produced by the worms of by bacteria in the exposures. The micrographs of control samples of EPA HW with HA sometimes showed a type of small (about 10 nm) particles (e.g., Figure 2.4A2a), probably representing the macromolecules of humic acid. Some of the micrographs from the exposures with ZnO-particles (e.g., Figure 2.4B2a) showed the

same type of small elongated particles that were found in the control samples. However, the micrographs from the exposures of both ZnO NPs (Figure 2.4B) and bulk particles (Figure 2.4C) were dominated by larger (about 10 nm to a few 100 nm) particles with oval, rounded or angular shapes, that occurred both as discrete particles and as part of larger aggregates. Some of those particles were surrounded by the diffuse 'matrix', presumably organic matter. The aggregates of ZnO-particles in the exposures (Figure 2.4) looked rather different from the aggregates of 'raw' ZnO particles (Figure 2.3), as they were not 'fused' together in the same way, but appeared to be composed of distinct particles. However, in most cases the size and shape factors of the aggregates in the different samples could not be shown to be significantly different (based on T-test with 5 % confidence level), neither when the samples of 'raw' particles were compared with the exposure media, nor when the different exposure media with different concentrations of ZnO particles (2.5 and 10 mg/L) and exposure media with and without humic acid were compared (Table 2.11). As previously stated, the number of aggregates found on the TEM-micrographs was probably too low to carry out a meaningful T-test. An exception from the other samples was the exposure sample with 2.5 mg/L ZnO NPs in EPA HW medium, in which the aggregates were significantly smaller and had significantly higher values of form factor, roundness, convexity and solidity compare with most other samples (Table 2.10). It is possible that a lower concentration of ZnO-nanoparticles, in the absence of HA, resulted in smaller and more compact aggregates. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

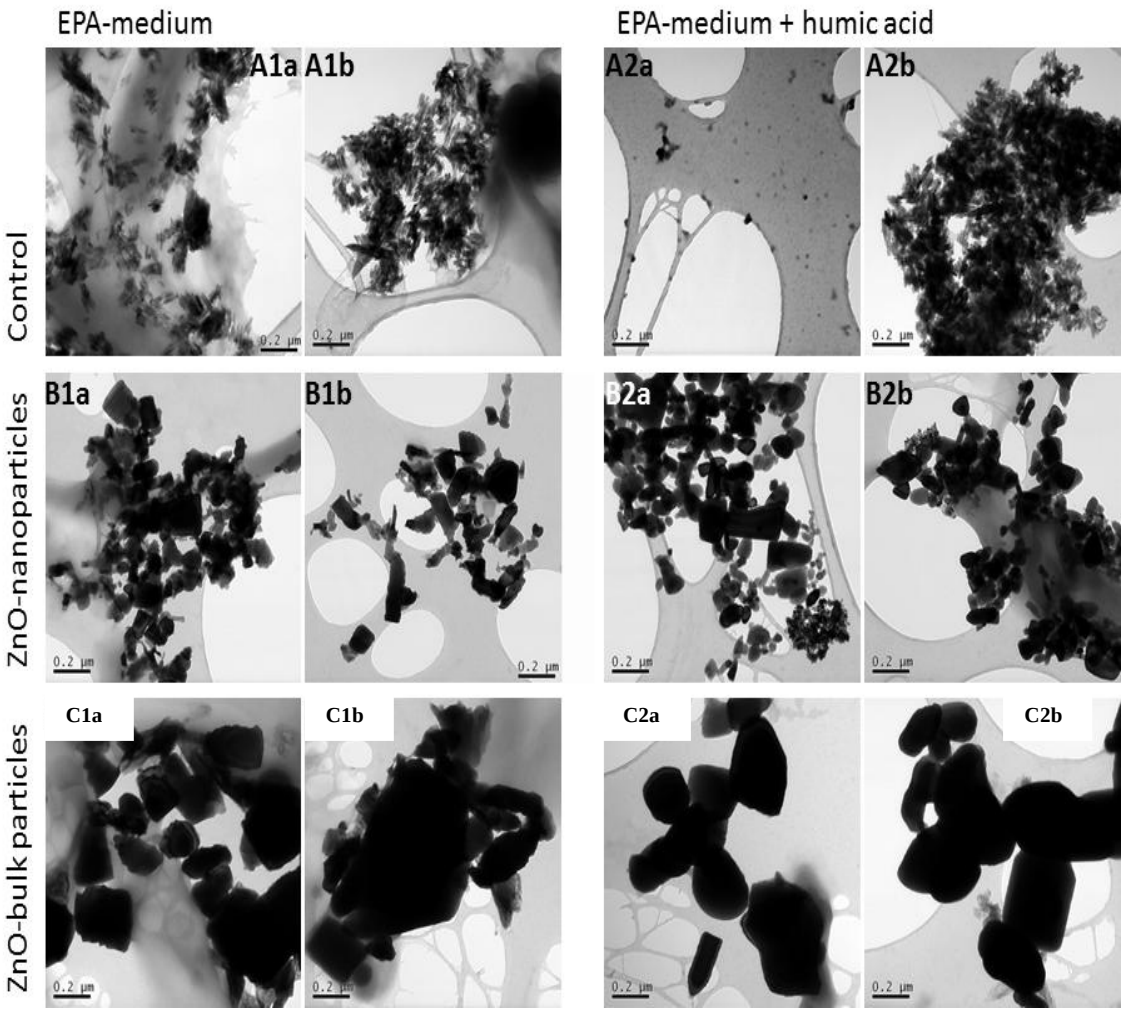

Fig. 2.4: TEM micrographs of exposure medium after 4 day worm-exposures. Control samples without ZnO particles (A), ZnO nanoparticles (B) and ZnO bulk particles (C) in EPA HW medium (A1, B1, C1) and EPA HW medium with humic acid (A2, B2, C2). (Lower case letters indicate image is from the same sample i.e. A1a and A1b are different images from the same TEM slide).

Table 2.12: Size and shape factors of particle-aggregates in medium after worm exposures (mean ± SD).

	ZnO-nanoparticles				**ZnO-bulk particles**			
	2.5 mg/L in EPA HW medium	10 mg/L in EPA HW medium	2.5 mg/L in EPA HW with humic acid	10 mg/L in EPA HW with humic acid	2.5 mg/L in EPA HW medium	10 mg/L in EPA HW medium	2.5 mg/L in EPA HW with humic acid	10 mg/L in EPA HW with humic acid
Length (nm)	430 ± 430	1140 ± 870	780 ± 910	630 ± 520	1650 ±	1770 ±	1100 ± 970	1720 ± 1220
Breadth (nm)	260 ± 300	720 ± 600	520 ± 730	380 ± 350	1080 ± 790	970 ± 600	690 ± 600	1220 ± 1060
Eq. circ.diam.	360 ± 370	950 ± 750	660 ± 830	520 ± 440	1400 ± 940	1420 ± 840	900 ± 770	1530 ± 1170
Form factor	0.4 ± 0.19	0.14 ±	0.2 ± 0.16	0.3 ± 0.20	0.23 ±	0.2 ± 0.16	0.3 ± 0.14	0.2 ± 0.20
Roundness	0.5 ± 0.14	0.4 ± 0.13	0.40 ± 0.099	0.4 ± 0.13	0.5 ± 0.14	0.3 ± 0.11	0.42 ± 0.098	0.36 ± 0.077
Convexity	0.7 ± 0.15	0.6 ± 0.10	0.6 ± 0.15	0.6 ± 0.17	0.6 ± 0.11	0.6 ± 0.15	0.7 ± 0.13	0.7 ± 0.21
Solidity	0.8 ± 0.15	0.6 ± 0.14	0.7 ± 0.13	0.7 ± 0.15	0.74 ±	0.7 ± 0.12	0.7 ± 0.11	0.6 ± 0.16

(Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

Sizes and shapes were also determined on the individual particles making up the larger aggregates. The TEM-micrographs from the exposure samples were used for this analysis. The results are shown in Figure 2.5 and Table 2.13. The size distributions were not normal, and are therefore described by the median value and the upper/lower quartiles (the average value coincides with the maximum of the size distribution only if the distribution is Gaussian). Both the discrete ZnO NPs and bulk particles had broad size distributions, but the nanoparticles were smaller (median equivalent circular diameter 91 nm) than the bulk particles (237 nm). The form factor and roundness of the ZnO-particles were around 0.6-0.7, and are consistent with most particles being oval in two dimensions (completely circular shapes have form factor and roundness of 1, lower values indicate deviations from circular shape). However, the bulk particles had lower form factor and roundness (median values 0.62 and 0.59 respectively) than the nanoparticles (median values 0.69 and 0.63), showing that the bulk particles were more elongated or angular in shape than the nanoparticles. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

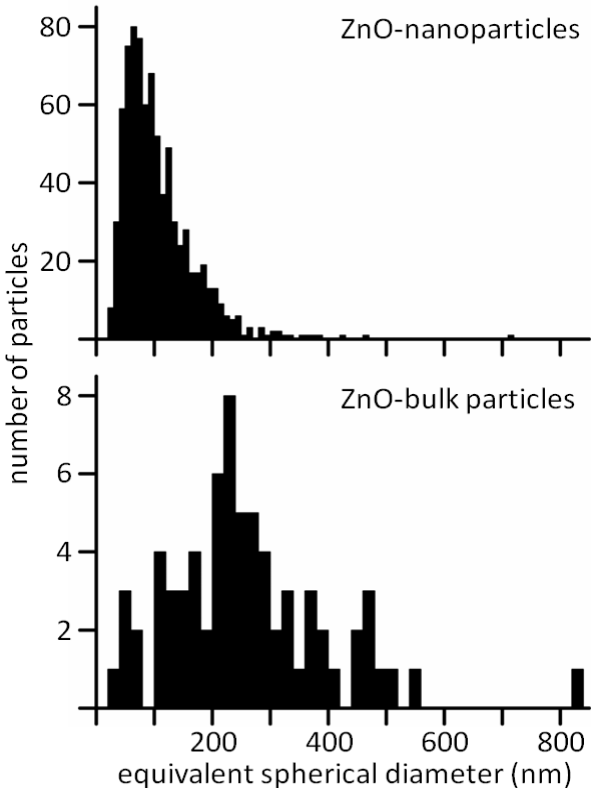

Fig. 2.5: Particle size (equivalent spherical diameter) of individual ZnO-nanoparticles and ZnO-bulk particles, determined by TEM.

Table 2.13: Size and shape factors of individual ZnO-particles, measured by TEM.

	ZnO-nanoparticles (n = 799)	**ZnO-bulk particles (n = 70)**
Length (nm)	108 ($^{159}_{74}$)	289 ($^{369}_{208}$)
Breadth (nm)	67 ($^{99}_{47}$)	175 ($^{218}_{122}$)
Equivalent circular diameter (nm)	91 ($^{133}_{64}$)	237 ($^{322}_{165}$)
Form factor	0.69 ($^{0.74}_{0.62}$)	0.62 ($^{0.68}_{0.55}$)
Roundness	0.63 ($^{0.72}_{0.52}$)	0.59 ($^{0.69}_{0.50}$)

Median (interquartile range) (n = number of particles measured)

AFM

Selected AFM images and diameter (height) distributions of particles determined by AFM (minimum 165 particles in each sample) are shown in Figure 2.6. In the sample of ZnO-nanoparticles in milli-Q water, only a few large particles could be found, and it was therefore not possible to determine the size distribution in that sample. For the other samples, the median sizes and upper/lower quartiles are given in Table 2.14, and they were considerably smaller than the sizes of individual ZnO particles determined by TEM. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

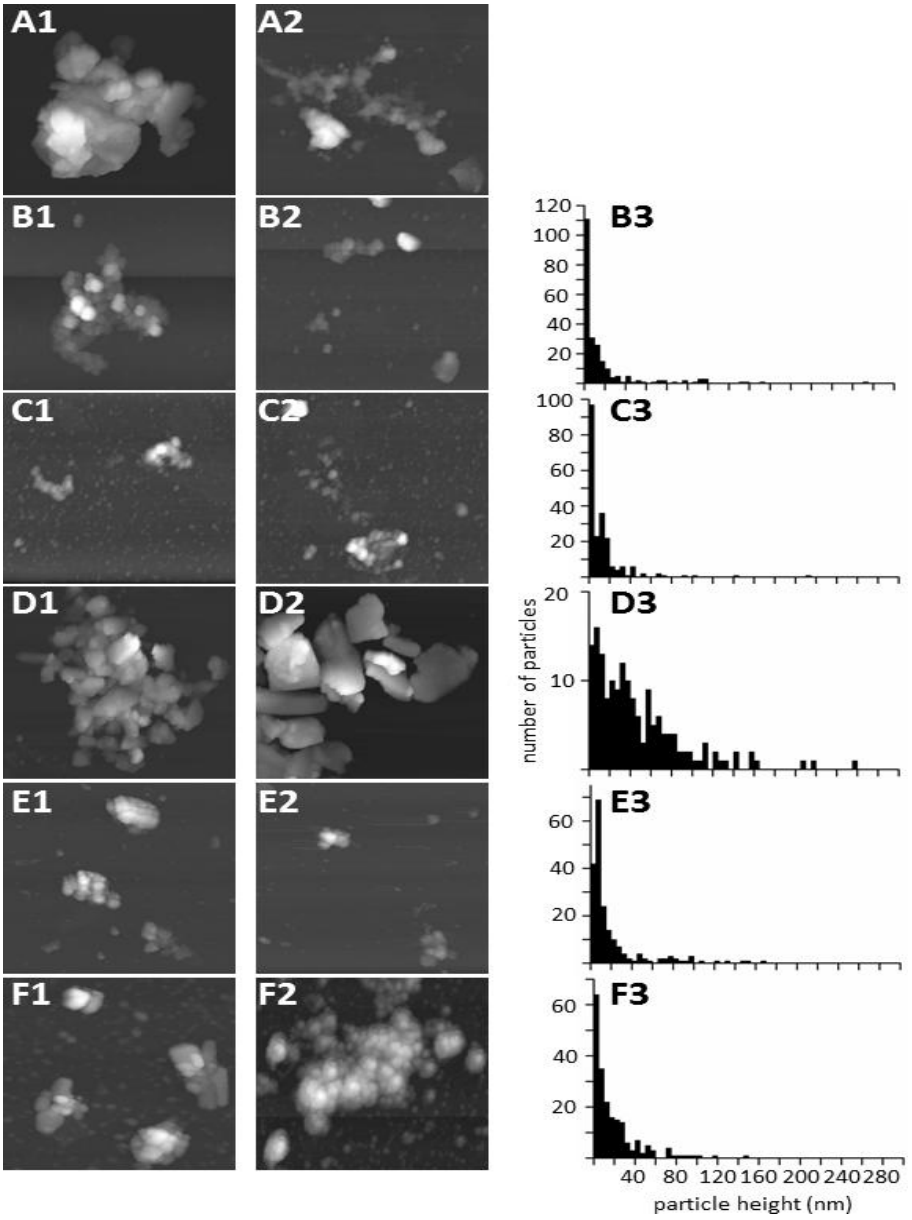

Fig. 2.6: AFM images (A1-F1, A2-F2) and associated particle height distributions (A3-F3) of ZnO-nanoparticles in milli-Q water (A), EPA HW medium (B) and EPA HW medium with humic acid (C), and of ZnO-bulk particles in milli-Q water (D), EPA HW medium (E) and EPA HW medium

Table 2.14: Median diameter (height) and interquartile ranges of ZnO particles in different media determined by AFM.

	ZnO nanoparticles	**ZnO bulk particles**
Milli-Q water		35.5 ($^{65.4}_{14.0}$) (n = 165)
EPA HW medium	5.2 ($^{16.2}_{3.3}$) (n = 231)	9.1 ($^{20.3}_{5.6}$) (n = 200)
EPA HW medium with humic acid	7.0 ($^{15.2}_{2.5}$) (n = 210)	10.8 ($^{25.8}_{4.4}$) (n = 204)

(Median height (interquartile range) (n = number of particles measured))

STEM-EDX

It was difficult to detect any levels of Zn above the background in the worm tissues exposed to ZnO particles using STEM-EDX. In one single worm from the sample of 10 mg/L ZnO bulk particles in EPA HW medium, Zn-rich particles with similar sizes and shapes as the ZnO bulk particles were found in the gut of the worm (Figure 2.7 a, b, c). Another type of nearly circular structures were found in the tissues surrounding the gut, and were rich in P and S (Figure 2.7 d, e), but without detectable levels of Zn.

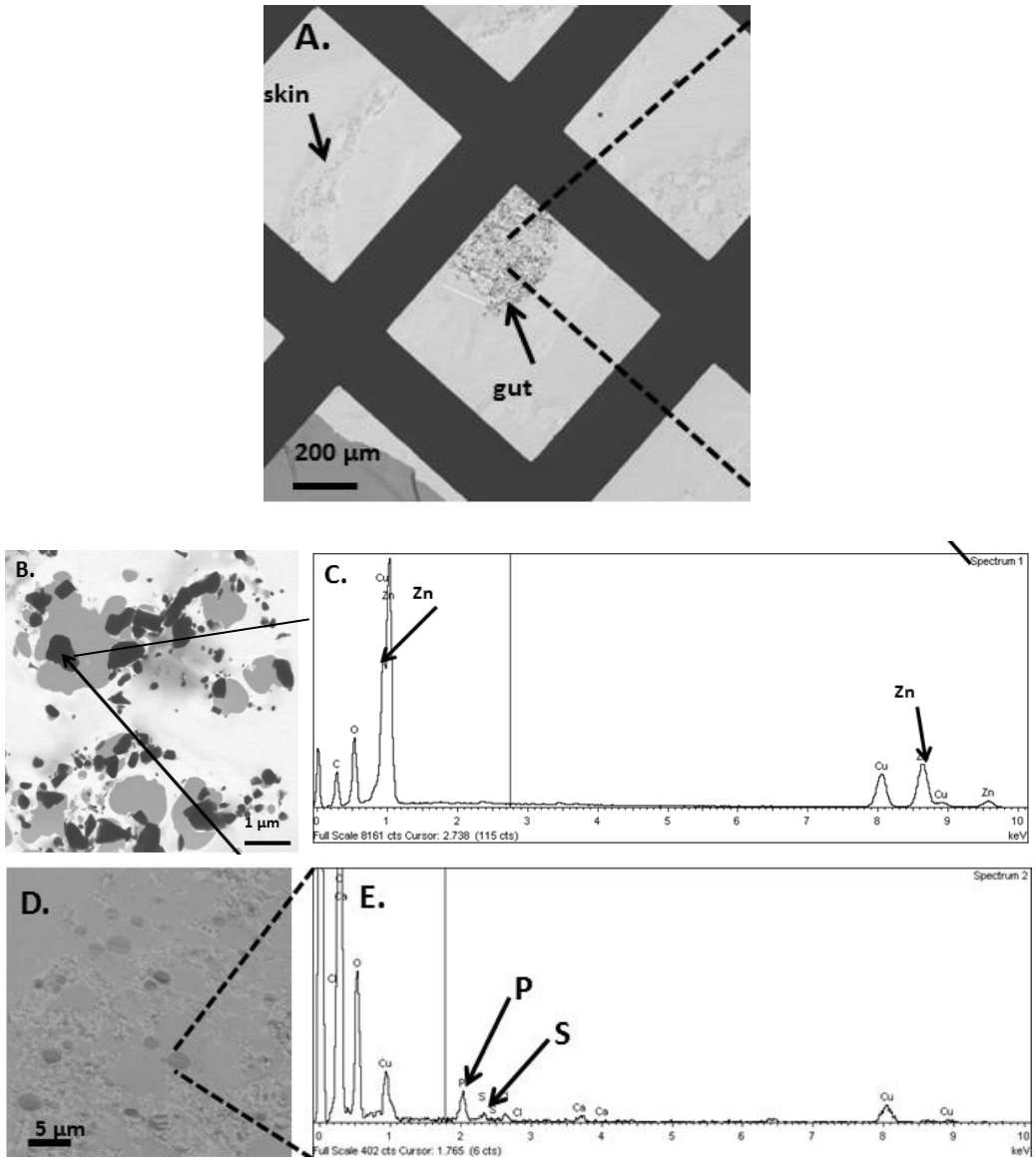

Fig. 2.7: STEM-micrographs (A, B, D) and associated EDX-spectra (C and E) from tissues of worms exposed to ZnO NPs and ZnO bulk particles for 10 days. Image B and spectra C show a Zn particle within the gut of the worm. Image D shows a particle in the gut however no Zn was detected (spectra E).

Dialysis

The containers outside the dialysis bags contained 10 mg/L ZnO particles, equivalent to 8.03 mg/L Zn. The concentrations of Zn in suspension immediately after the start of the experiment was 5.49 mg/L for nanoparticles in EPA HW medium, 7.71 mg/L for nanoparticles in EPA HW medium with humic acid, 7.83 mg/L for bulk particles in EPA HW medium, and 5.47 mg/L for bulk-particles in EPA HW medium with humic acid. In the end of the dialysis experiment (after 33 days) the concentrations of suspended Zn had decreased to 2.75 mg/L for nanoparticles in EPA HW medium, 2.44 mg/L for nanoparticles in EPA HW medium with HA, 1.64 mg/L for bulk-particles in EPA HW medium and 1.51 mg/L for bulk particles in EPA HW medium with HA. The concentrations of Zn inside all the dialysis bags increased rapidly immediately after the start of the experiments (Figure 2.8).

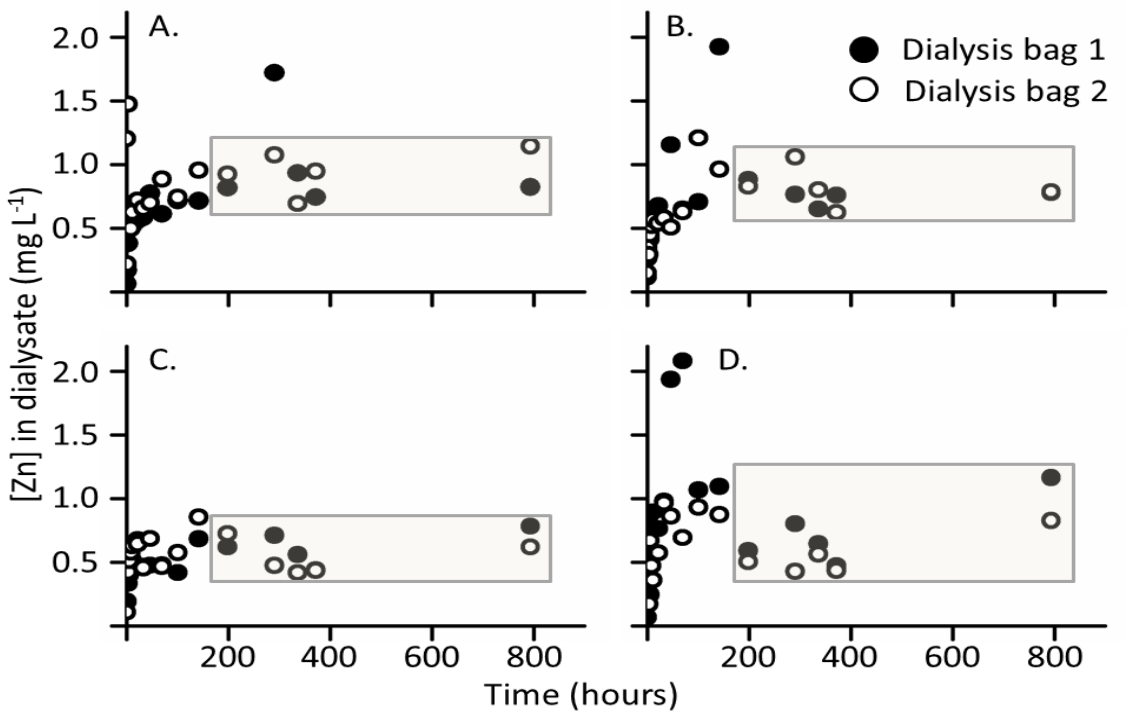

Fig. 2.8: Variations in Zn-concentration with time inside dialysis bags immersed in 10 mg/L suspensions of ZnO-nanoparticles (A, B) and ZnO bulk particles (C, D) in EPA HW medium (A, C) and EPA HW medium with humic acid (B, D). Data points used for calculating the solubility are indicated by the filled squares.

The increase in Zn concentrations became successively slower, and the concentrations usually stabilised after around 200 hours. The average Zn concentrations inside the dialysis bags after 198 hours (including all but one outlying data point in the ZnO NP sample in EPA HW medium, Figure 2.8A) were 0.9 ± 0.15 mg/L for nanoparticles in EPA HW medium, 0.8 ± 0.12 mg/L for nanoparticles in EPA HW medium with humic acid, 0.6 ± 0.13 mg/L for bulk particles in EPA HW medium, and 0.6 ± 0.23 mg/L for bulk particles in EPA HW medium with humic acid. There was a significant difference in solubility between nanoparticles and bulk particles in EPA HW medium (based on T-test at 5% significance level). There was no significant difference between the solubility of NPs compared with NPs with HA, between bulk particles and bulk particles with HA and between NP with HA and bulk particles with HA.

2.3.4 Summary of results

Table 2.15 below shows a summary of the results found in the studies from HWU and FENAC.

Table 2.15: Summary of results obtained from studies performed at HWU and FENAC.

	NP	**NP + HA**	**Bulk**	**Bulk + HA**
Manufacturer Size	40-100nm	-	-325 mesh (~44µm)	-
DLS HD Diameter (DI water @ 0.01mg/L)	406.7nm	372.3nm	1436nm	1228nm
DLS HD Diameter (EPA medium @ 0.01mg/L)	1021nm	939.6nm	1924nm	2272nm
Zeta Potential (DI water @ 0.01mg/L)	-31.3	-34.1	-38.5	-43.0
Zeta Potential (EPA medium @ 0.01mg/L)	-29.5	-30.3	-34.6	-34.7
BET Surface Area	$12.064 m^2 g^{-1}$	-	$5.821 m^2 g^{-1}$	-
XRD Crystallite Size	199 ± 53nm (confirmed ZnO)	-	208 ± 28nm (confirmed ZnO)	-
TEM (agglomerates)	720 ± 300nm	530 ± 740nm	730 ± 390nm	730 ± 840nm
TEM (NPs) (Median(interquartile range))	91 (133/64)nm	-	237 (322/165)nm	-
Dialysis	0.9 ± 0.15 mg/L	0.8 ± 0.12 mg/L	0.6 ± 0.13 mg/L	0.6 ± 0.23 mg/L
AFM (Median(interquartile range))	5.2 (16.2/3.3)nm	7.0 (15.2/2.5)nm	9.1 (20.3/5.6)nm	10.8 (25.8/4.4)nm

2.4 Discussion

The physicochemical properties of NPs have been extensively referred to in the literature as important factors in determining their potential toxicity (e.g. Adams *et al*., 2006; Xia *et al*., 2008; Cho *et al*., 2011). As mentioned in the introduction, in order to sustain the nanotechnology sector, comprehensive studies must be conducted to assess the toxicity of NPs in relation to environmentally relevant organisms and a major part of such studies will entail characterising and understanding the physicochemical properties of these NPs.

2.4.1 DLS Study

Time course 96 hour study

A number of studies have investigated the agglomeration of NPs in aqueous suspensions and most nanoparticles have been shown to agglomerate when hydrated (Chen *et al*., 2007; Pettibone *et al*., 2008; Berg *et al*., 2009; Domingos *et al*., 2009, Keller *et al*., 2010). These studies investigated numerous conditions, such as pH, concentrations of organic matter and the ionic strength of the suspensions, which can affect the agglomeration of particles and ultimately their fate in environmental media. In the present study, the effects on agglomreation of salts ($CaSO_4$, $MgSO_4$, $NaHCO_3$ and KCl) within a medium, the effects on agglomeration of organic matter and the effects on agglomeration over time were investigated. pH was not considered in these studies due to size (e.g. space, access to DLS machine), cost (Suwannee River HA is very expensive) and time constraints. Within this study contaminant particles were detected in all four control media (deionised water with and without 5mg/L HA and EPA HW medium with and without 5mg/L HA) at each time point during the 96 hours study and this was to be expected as the media had not been filtered as recommended by the user manual for the Malvern Zetasizer (2008). The reason the media had not been filtered was for the data to give a relevant representation of the media used in ecotoxicological experimentation. However, the media had been filtered in the size measurement experiment and contaminant particles were still detected in the controls. This suggests that the 0.2µm filter may not have been sufficient to remove contaminant particles (e.g. constituent salts in the EPA HW media and dust particles in both media types). The recommended minimum concentration suggested by Malvern for particles

of 10-100nm is 0.1mg/ml, or for 100nm-1µm is 0.01mg/ml. The particles used in this study, according to the manufacturer, ranged from 40-100nm (NP) and -325mesh (~44µm; bulk) and so the highest experimental concentration of 10mg/L may have proved to be the most relevant. However, the Malvern manual also states that various concentrations should be investigated to avoid concentration dependent effects. In this study each factor, i.e. the concentration of particles, time, the medium type and the particle type, was seen to have a significant impact on the results when using DLS to characterise the HD and ZP of ZnO NPs in environmentally relevant media. The recommended minimum concentration of NPs, that is 0.01mg/ml, was found to yield the only significantly different HD results from the control. When measuring ZP however this is not the case as all concentrations were found to have various degrees of stability. This is not an unexpected result as the Zetasizer will measure ZP at any concentration provided there is a count rate of 20kcps or above. As expected, it also appears that this study is time sensitive as 0 hours was significantly different from all other time points. This may be attributed to the solubility of ZnO NPs and their tendency to aggregate/agglomerate. The media type was also found to be a significant factor. It was expected that the EPA HW suspensions would have greater hydrodynamic diameters than the sterile water suspensions as the salt concentrations of the media would increase agglomeration and that humic acid would increase the dispersion and stability of the suspensions. This study suggests that the NPs interact with salts in the media and with organic matter. HA was found to increase the stability of the suspensions in both media types, which is in keeping with the literature as in previous studies humic substances have been shown to increase the dispersion and stabilise suspensions of nanoparticles (Chen and Elimelech, 2006; Hyung *et al*., 2007).

As mentioned in the introduction, DLS is a useful method for assessing the size and stability of a suspension of nanoparticles, however cautious interpretation of the results is necessary. It is clear from the results obtained in this study that it is necessary to examine numerous parameters when exploring the data released from the Malvern Zetasizer Nano. Often publications will cite the Z-average number (the hydrodynamic diameter) without a reference to the e.g. PdI, count rate or intercept, all of which give meaning to the Z-average value. According to the Malvern User Manual (2008) there are a number of parameters that must be within a certain range in order for a reading to be “acceptable”. These parameters include having a count rate 10 kcps (kilo counts per

second) in excess of the media, the PdI (polydispersity index) should be less than 0.5 (0.7 quoted in other editions of the manual) and the intercept should be between 0.85 and 0.95 for a "good" reading. When a sample produces a result that is outside of these parameters it will suggest numerous reasons as to why this may be in the "expert report". These reasons can include a sedimenting or aggregating sample, an unstable suspension or measurements deviating from one another, amongst others. As mentioned in the results very few of the samples fitted within the required parameters of the Zetasizer. I feel this does not, however, mean that this method is not suitable however the data need to be interpreted with care, not as individual numbers but as patterns over time.

Size measurement study

In the size measurement study the factors of concentration, media type and particle type were seen to be significant in determining size. In the HD measurements the controls were found to have significantly smaller sizes that 0.1 and 0.01mg/L ZnO as expected. The media type was also seen to be significant with EPA HW with HA having a significantly higher HD that DI with HA, again as expected due to the presence of salts in the EPA HW with HA media. Finally, the NPs were found to have significantly lower HDs than bulk particles. These results were reflected in the ZP study as the controls were found to be less stable than other concentrations due to the lack of particles in the controls. The media was also seen to be a significant factor and the HA was seen to increase the stability of the suspensions in keeping with current literature. Finally, the particle factor was found to be significant with bulk suspensions being significantly more stable than the NPs. This may have resulted from the solubility of the particles as the solubility of the NPs was found to be significantly higher than that of the bulk particles in the dialysis study. When comparing the effects of filtering the media prior to testing it was seen that filtering the media had little effect on the hydrodynamic diameter of the nanoparticles but increased their stability and within the bulk studies filtering the media increased the consistency of the hydrodynamic diameter across the concentrations but did not reduce the size of the hydrodynamic diameter. This suggests that, at least for this particle type, filtering does not significantly influence the readings.

2.4.2 FENAC Studies

BET

The surface area of the NPs was found to be much greater than that of the bulk particles in powder form. This was to be expected because as size decreases the surface area reactivity increases (Christian *et al*., 2008). A number of papers in the literature have stated that the greater the surface area of a particle, the greater the reactivity of that particle and in turn the greater the potential for that particle to have an increased toxicity (e.g. Pal *et al*., 2007; Navarro *et al*., 2008; Li *et al*., 2006).

XRD

Within the XRD study, the bulk crystallite diameter was smaller than the average circular diameter determined by TEM. This result is expected, since the crystallite diameter should be equal or smaller than the particle size. However, for NPs, the crystallite diameter was approximately double the circular diameter determined by TEM. This unexpected result could perhaps be explained by the fact that the NPs were highly polydispersed, as indicated by the DLS study. Larger particles can dominate the XRD-signal (Calvin *et al*., 2005), resulting in a misrepresentation of the sample. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

AFM

In a number of samples, the median sizes and upper/lower quartiles were considerably smaller than the sizes of individual ZnO particles determined by TEM. This finding can be explained by the fact that AFM cannot distinguish between particles of different composition and it is therefore possible that particles other than ZnO (e.g. HA macromolecules or precipitates from the salts of the EPA HW medium) are included in the particle size distributions determined by AFM. Similarly, the much smaller median size of the ZnO bulk particles in EPA HW media (with and without HA) compared within milli-Q water, could be explained by the presence of particles other than ZnO (e.g., HA macromolecules or salt precipitates), by the formation of other Zn-rich

particles following the dissolution of ZnO, or by the disaggregation of ZnO particle aggregates (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

TEM

The TEM images indicated that the particles were several hundred nm in diameter, appearing to be composed of smaller NPs that were aggregated or "fused" together. These aggregates were not circular, were highly branched and concave. The images showed no significant difference between NPs dispersed in EPA HW and EPA HW with HA but the testing was not 100% robust due to the sample size. This result is in keeping with the dissolution study where HA had no significant impact on the dissolution of particles. However, as stated in the DLS study, the addition of HA gave stability to the dispersion. This stability may be derived from the diffuse "matrix" that was seen to surround the particles, which was presumed to consist of organic matter.

STEM-EDX

As mentioned in the results section it was difficult to detect any zinc above background levels in the worm tissues which had been exposed to various concentrations of ZnO NPs and bulk particles. As zinc was found in the gut of at least one worm it can be concluded that the worms do ingest the particles. The reason that it was difficult to find zinc in any other sample may have been because the sections of tissue were extremely thin (~100nm) and so the particles may not have been sampled in the subsection of worm examined. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

Dialysis

Understanding the aquatic chemistry of NPs may also be an important consideration when investigating the toxicity of these NPs. Concerns have arisen in the literature about the bioavailability of NPs after they enter the environment. The solubility of ZnO is highly dependent on the pH of the solution it is in and it has been reported to be anywhere in the range of several thousand mg/L at pH 6 to approximately 1mg/L at pH 8 (Stumm and Morgan, 1995). The rate of dissolution is proportional to the particle

surface area and so NPs should dissolve at a faster rate than their bulk counterparts (Borm *et al*., 2006). In addition to this, the size of the particle may also be relevant when discussing the solubility, with NPs expected to have higher equilibrium solubility than bulk particles (Borm *et al*., 2006). Our traditional understanding of equilibrium solubility for bulk materials is challenged by the additional complexities of working with nano-sized materials (Franklin *et al*., 2007). The solubility of the NPs and bulk was determined in this study as 0.9 ± 0.15 mg/L and 0.8 ± 0.12 mg/L, for NPs in EPA HW and EPA HW with HA and 0.6 ± 0.13 mg/L and 0.6 ± 0.23 mg/L for bulk particles in EPA HW and EPA HW with HA, respectively, provided that the aggregation/adsorption of ZnO NPs and bulk particles did not influence the solubility, that equilibrium between ZnO and soluble Zn was reached after 198 hours, and ZnO-particles did not pass through the 1 kDa membrane. This solubility was found to be in agreement with the previously mentioned literature as NPs were found to have a significantly higher equilibrium solubility after 200 hours. It was also observed that there was a rapid dissolution of ZnO particles outside the dialysis bags however the speed at which NPs dissolved was not investigated in comparison with the bulk particles. The media type also influenced the dissolution of the ZnO as in the case of NPs in EPA HW and EPA HW with HA media there was considerable aggregation or adsorption of particles onto the container walls, which had taken place immediately after their addition to the media. After 33 days, 61-88% of the ZnO had aggregated or adsorbed onto the container walls. (Report FENAC/2010/2012/003, Dr. Bjorn Stolpe).

2.4.3 Comparison of results obtained at Heriot Watt University and at FENAC

When comparing the DLS HD data obtained at HWU and the TEM data obtained from FENAC it can be seen that the NP HDs are in the same range but the bulk particles are not, in both EPA HW medium and EPA HW medium with HA. The DLS technique found the bulk particle to be much larger in HD than the TEM technique. This discrepancy can be explained by the fact that the TEM investigated individual particles and agglomerates separately. The DLS result can be influenced by the polydisperse nature of a sample whereas the TEM is not. Furthermore, TEM was able to establish that there was a broad distribution of particle sizes but that the NPs (91nm) were smaller than the bulk particles (237nm) in median value. This comparison of results confirms

that a technique such as TEM may be more useful in determining the size of an NP in environmental media than DLS.

Chapter 3 Acute Toxicity Testing: Investigation of the effects of ZnO nanoparticles and bulk particles on the behaviour of *Lumbriculus variegatus.*

3.1 Introduction:

As mentioned in chapter one, *L. variegatus* have been extensively used to test the toxicity of a variety of contaminants. Most oligochaete species are likely to be found at the interface between sediment and water or, in more shallow habitats, at the interface between water and air, and so they will often encounter steep environmental gradients and abrupt transitions when exposed to chemical or biological factors that may affect their rate of survival (Drewes, 1997). The behaviours of *L. variegatus* are highly stereotyped and so are very useful as a biomarker for toxicity.

3.1.1 The nervous system and functions of locomotor behaviours in Lumbriculus variegatus

The behaviours of helical swimming and body reversal in *L. variegatus* were first described in detail by Charles Drewes in the 1990s (Drewes, 1997; 1999). Locomotor behaviours are integrally linked with the worm's ability to forage, sexually reproduce, avoid predators, disperse quickly and react to general environmental cues (Drewes, 1997). Helical swimming and body reversal behaviours are most likely anti-predation strategies, used both singularly or in combination, throughout the post embryonic life of the worm. Drewes (1999) hypothesised that since *L. variegatus* tend to feed *en masse*, when a negative stimulus occurs the worms will execute body reversal behaviours which rapidly disaggregate the assembled mass and this can confuse or startle a predator. The body reversal behaviour positions the head of the worm away from the predator or negative stimulus and swimming behaviour allows for rapid, short distance retreats from threats, where the worm doesn't have the benefit of protection or traction (Drewes, 1999).

The gross anatomy of *L. variegatus* was first described by Isossimow (1926), with an English summary in Stephenson's book "The Oligochaeta" (1930). The central

nervous systems of *L. variegatus* consists of a cerebral ganglion (or "brain"), which is located in the first segment of the worm and a ventral nerve cord that extends along the length of the body into each segment. In each segment, except the first two, the ventral nerve cord splits into four pairs of segmented nerves. The segmented nerves extend laterally into the body wall (Stephenson, 1930). Some of these fibres act upon stimulation of the body surface and other have motor functions and innervate the circular and longitudinal muscle. Rapid escape behaviours occur when the worm rapidly withdraws in response to an abrupt stimulus. This stimulation excites segmental nerves to conduct activity centrally to the ventral nerve cord, where sensory fibres make synaptic connections with a central neurophil (Drewes, 1984). Photoreceptors in the tail of the worm detect shadows and these trigger rapid shortening of the tail (Drewes and Fourtner, 1989). Swimming in *L. variegatus* is always stimulus evoked and brief in length. For this reason it is unlikely that this behaviour is a means of sustained vertical movement or horizontal migration. One possible benefit to swimming is the potential for the worms to be transiently planktonic (i.e. drift in the water column to exploit food sources if necessary) and allow them to quickly escape adverse conditions or exploit available resources (Drewes, 1999).

Since these behaviours are so well documented and are also highly stereotyped they are amenable to quantification (Rogge and Drewes, 1993; Ding *et al*., 2001). The stereotyped behaviour of *L. variegatus* make them a useful tool in detecting and investigating the sublethal effects of environmental contaminants using a touch stimulus assay referred to from here on as the "head flick" assay.

3.1.2 Aims of the study

The aim of this study was to investigate the effects of ZnO NPs and bulk particles on the mortality and body reversal behaviour of *Lumbriculus variegatus* in water only (i.e. no sediment) acute toxicity tests. The use of water only tests is relevant in this species as they are capable of moving within the water column and so may come in contact with potential contaminants held within the water column. A further aim was to assess the impact of organic matter on the toxicity of ZnO NPs and bulk particles. These tests were conducted over 96 hours. The null hypotheses proposed prior to the beginning of the study were that:

1. Feeding of the worms during physiological synchronisation has no effect on the behaviour of the worms during testing with ZnO NPs.
2. NPs and bulk particles have no effect on the behavioural response of the worms after a 96 hour water only exposure at the concentrations tested.
3. The addition of 5mg/L humic acid has no effect on the behavioural response of worms exposed to NPs and bulk particles after a 96 hour water only exposure at the concentrations tested.
4. The interaction of the concentration factor and the humic acid factor is not statistically significant in the effects of the NPs/bulk particles after a 96 hour water only exposure at the concentrations tested.

3.2 Methods

These studies were carried out at two locations, the Technical University of Denmark (DTU) in Lyngby, Denmark and at Heriot Watt University (HWU) in Edinburgh, Scotland.

3.2.1 United States Environmental Protection Agency medium (EPA HW)

The medium the worms were exposed and maintained in was USEPA hard reconstituted water (EPA HW) as described in Chapter 2, Section 2.2.1.

3.2.2 Maintenance of the Lumbriculus variegatus cultures

At DTU, the *L. variegatus* cultures were maintained according to a protocol devised within the Nano Safety group at HWU and that was adapted from the OECD Guideline 225 (2007). The cultures were stored in 2 x 10L aquarium tanks in a temperature controlled room at 20 ± 2°C with a light regime of 16:8 hours at 100 - 500 lux. The substrate used within the tanks was cut strips of unbleached (not autoclaved) brown paper towels. The culture medium was renewed once per week by removing the aquarium lids and slowly pouring out the overlying water from the aquarium. The worms and paper towels were transferred to a clean container containing EPA HW

medium. Any paper towels that appeared discoloured were removed. The culture tanks were then scrubbed with a sponge in deionised water. When the aquarium was clean the worms and paper towels were transferred back into it. The paper towels were replenished if necessary. The culture aquarium was then topped up with approximately 5L of culture medium. Following this 0.7g of pre-powdered aquaria tropical flake food ("TetraMin", Tetra) was added to each aquarium. The aquaria (with tank lids) were replaced in the temperature controlled room with aeration via an air pump (Eheim Air Pump 100) attached to aquarium tubing (Algarde Silicone Tubing 6mm), filters (0.3µm 50mm diameter, Whatman) and 200µl pipette tips.

At HWU, the *L. variegatus* cultures were maintained according to a protocol that was adapted from the OECD Guidelines 225 (2007). The cultures were stored in 2 x 10L tanks in an incubator at 20 ± 2°C with a light regime of 16:8 hours at 100 - 500 lux. The substrate used within the tanks was cut strips of unbleached brown paper towels (which were prewashed and autoclaved). The culture medium was renewed once per week by sieving the paper towels through a 500µm mesh into a large plastic tub. The culture tanks were then scrubbed in tap water and rinsed in culture medium. The paper towels were replenished if necessary and the worms were rinsed from the sieve, using culture medium, back into the clean tanks. Worms were also collected from the bottom of the plastic tub using a pastette and returned to the tanks. The culture tank was then topped up with approximately 5L of culture medium. Following this 0.7g of pre-powdered aquaria tropical flake food ("TetraMin", Tetra) was added to each tank. The tanks (with tank lids) were replaced in the incubator with aeration via an air pump (Hailea Super Silent Adjustable Air Pump) attached to aquarium tubing (Algarde Silicone Tubing 6mm) and glass pipettes.

3.2.3 Equipment

At DTU, each item of glassware was acid washed using a Miele Professional G7835 CD Glassware Washer. The machine was programmed for 1.25 hours and the steps included a pre-wash, cleansing, neutralisation, rinses, final rinse and drying. At HW, each item of glassware was acid washed by hand in a 50% (v/v) 5M HNO_3 solution.

Glassware was left in acid for 15 minutes and after was rinsed four times in distilled water. Items were allowed to air dry.

3.2.4 Physiological synchronisation of worms prior to testing

Twelve days prior to testing worms were artificially fragmented according to a protocol adapted from the OECD Guideline 225 (2007) as described in chapter 2, section 2.2.2.

3.2.5 ZnO ecotoxicity testing – preliminary work

Pilot studies were conducted in order to find a suitable range of sublethal doses due to the lack of exposure data available. A wide range of doses were chosen based on available NP and bulk ZnO exposure literature which used a number of environmentally relevant species. These species included *Daphnia magna* (e.g. Adams *et al*., 2006; Heinlaan *et al.*, 2008; Blinova *et al*., 2010), *Danio rerio* (e.g. Zhu *et al*., 2008), *Pseudokirchneriella subcapitata* (e.g. Franklin *et al.*, 2007; Aruoja *et al*., 2008), micro-organisms (e.g. Adams *et al*., 2006; Huang *et al*., 2008; Mortimer *et al*., 2008) and plants (e.g. Lin and Xing, 2008). As a result of these pilots the dose range chosen was 0, 1.25, 2.5, 5 and 10mg/L ZnO NP and bulk. The effect of organic matter on the toxicity of the particles was investigated and 5mg/L Suwannee River Humic Acid (HA) was chosen to reflect what was environmentally relevant, based on the available literature (e.g. Collins *et al*., 1986; Zhang *et al*., 2009) and previous projects within the research group, conducted by Jon Mullinger, as described in Chapter 1, Section 1.4. Pilots were also conducted in order to assess the best method of husbandry after physiological synchronisation of the worms and positive controls of $ZnSO_4$ and $CuSO_4$ were used to confirm the test protocol was valid. O'Gara *et al.* (2004) showed that $CuSO_4$ was toxic to *L. variegatus* over various time points in a seven day period. It has also been shown that $ZnSO_4$ is toxic to a variety of oligochaetes (Callahan *et al*., 1994; Reinecke *et al*., 1996). The doses chosen for this study were based on the sublethal doses observed in O'Gara *et al*. (2004) and Callahan *et al*. (1994).

3.2.6 Pilot study: Assessing the effect of particle exposure on fed vs. not fed worms

This study was carried out at DTU, Lyngby, Denmark. Worms were synchronised as described in Chapter 2, Section 2.2.2. During synchronisation in this pilot study 200 worms were kept in two separate 500ml beakers (100 worms per beaker) containing 350ml EPA HW medium and shredded unbleached paper towels (un-autoclaved). One beaker was fed at day 7 and one was not. The ZnO NPs were sourced from Alfa Aesar, Germany. Once weighed the NPs were transferred to a volumetric flask containing EPA HW medium and were sonicated in a sonication bath (Ultrawave Q series, 400W) for 30 minutes. Solutions of 1.25mg/L, 2.5mg/L, 5mg/L and 10mg/L ZnO NP were made up in EPA HW medium from the sonicated stock. When the NP suspensions were prepared the temperature, dissolved oxygen and pH were measured (Table 3.1).

Table 3.1: Parameters tested during exposure

Parameter	**Level**	**Checked**
Temperature	20°C ± 2°C	1 replicate at the beginning and end of testing
Dissolved oxygen	> 30% saturation	1 replicate at the beginning and end of testing
pH	6.0 – 9.0	1 replicate at the beginning and end of testing

The dissolved oxygen was measured with a Hach LDO HQ10 O_2 dissolved oxygen meter. The temperature was also measured using the DO meter which was inserted into the medium and gently stirred for 30 seconds until the temperature stabilised. The probe was allowed to run until the percent oxygen saturation stabilised. The pH was measured using a pHM210 standard pH meter (MeterLab Radiometer Analytical). The probe was inserted into the medium and it gently stirred in the medium until the pH stabilised. After determination of each suspension's parameters 20ml of the required concentration was added via a 20ml syringe to each glass vial. After the suspension was added one worm was added per vial. The vials were then moved to the 20°C ± 2°C controlled temperature room and arranged at random under a light regime of 16:8 hour light:dark. They were left in the controlled temperature room for 96 hours. During the 96 hour period vials were observed for any obvious changes in behaviour (e.g. spasms in the worms) when compared to the controls or any mortalities. Any observations were recorded.

Upon completion of the 96 hour period in the controlled temperature room the vials were removed back to the laboratory. Vials were checked for mortality. Mortalities were assessed and recorded under the following conditions:

- no sign of movement after gentle stimulus;
- decomposition was apparent;
- no worm in the vial (i.e. complete decomposition).

Following this, the "head flick" assay was performed. Worms were gently removed from the vials using a pastette and transferred to a glass petri dish along with some liquid from the vial to allow the free movement of the worm. The assay was carried out according to Drewes (1999). Touch stimuli were delivered via the tip of a pastette. The anterior portion of the worm was gently touched to evoke a behavioural response. The flexibility of the pastette tip ensured the worm was not injured during stimulation. The worm was touch stimulated a total of ten times and the behavioural response was recorded in terms of the total number of body reversal movements that occurred. This body reversal can be seen in figure 3.1 below (taken from Drewes, 1999). To avoid observer bias only completed 180° turns were accepted to be counted. The results of this study were used to determine whether worms in subsequent tests should be fed during regeneration.

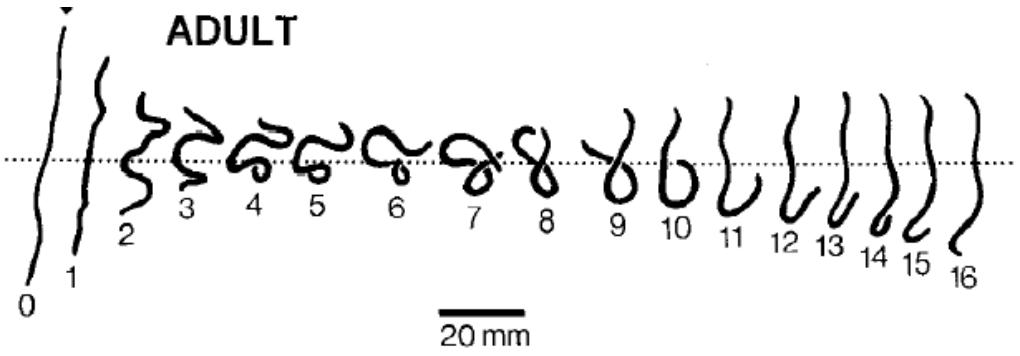

Figure 3.1: The body reversal behaviour of *Lumbriculus variegatus* (Drewes, 1999).

The results were analysed statistically using PASW software 17.0. Data were normalised to the control behavioural response by dividing the percent behavioural response by the mean of each test and multiplying by 100. The data were then tested for normality using a Kolmogorov-Smirnov test. Since the data were found not to comply with parametric tests requirements, a Scheirer-Ray-Hare test, equivalent to a two-way ANOVA (Dytham, 2011). The Scheirer-Ray-Hare test is often used in animal behaviour studies (e.g. Tolimeiri *et al*., 2000; Tosh *et al*., 2006; Todd *et al*., 2011; de la Fuente *et al*., 2012) where the data does not meet the assumptions of normality. This was followed by Mann Whitney tests, which assessed where any differences between the treatments may lay. A Bonferroni correction was applied to account for the use of multiple tests. In summary, the standard significance threshold (0.05) was divided by the number of multiple tests conducted and significance accepted only for P values below this new threshold. Although this is a conservative correction it reduces the probability of making a type I error (rejecting the null hypothesis (NH) when it should be accepted).

3.2.7 Nanoparticle and bulk studies

These studies were carried out at both DTU (Denmark) and HWU (Scotland). Twelve days prior to testing worms were artificially fragmented according to the protocol as described previously in Chapter 2, Section 2.2.2. The same concentrations of bulk particles and NPs were used as described for the feeding test in Section 3.2.5, both with and without the addition of 5mg/L Suwannee River humic acid (HA). HA was added to the dilutions after sonication (as described in Chapter 1, Section 1.5.2). At DTU, when the nanoparticle and bulk concentrations were prepared the temperature, dissolved oxygen and pH were measured and the suspensions and worms were added to the vials as described previously in Section 3.2.5. The test set up at Heriot Watt was the same as the set up at DTU except that an incubator at 20 ± 1°C with a light regime of 16:8 hours was used as opposed to a temperature controlled room; in addition the NP study at DTU was n = 3, the bulk study at DTU was n = 1, whereas the NP and bulk study at HWU was n = 3.

Upon completion of the 96 hour period in the controlled temperature room the vials were removed from incubation and assessed for mortality and behavioural response as described previously in section 3.2.6. After assessing the vials for mortality and behavioural response the worms were preserved in Bouin's solution for 4-5 hours before being transferred to 70% ethanol ready for processing for histology.

3.2.8 Positive control studies

Two positive controls were set up using $CuSO_4$ at 0.2µM and 0.4µM and $ZnSO_4$ at 2.5mg/L and 10mg/L, in the presence and absence of 5mg/L HA. These tests were set up in the same way as the nanoparticles and bulk studies (except without sonication), with n = 3. The results were statistically analysed using PASW 17.0 software. Data were normalised to the control behavioural response. Normality was investigated using a Kolmogorov-Smirnov Test. Non parametric data was investigated for significance using a Scheirer-Ray-Hare test followed by Mann Whitney tests as described previously in Section 3.2.6. A bonferroni correction was applied to account for the use of multiple post hoc tests. Data found to comply with parametric tests requirements were analysed using a 2 way ANOVA, followed by post hoc Tukey tests.

3.3 Results

3.3.1 Experimental design for each study

Table 3.2 below shows the experimental design for each study in this chapter. The p value was accepted at 0.05, unless a Bonferroni Correction was applied (as detailed in each section).

Table 3.2: Experimental design for the studies within this chapter

Experiment	Test	Factor	Factor	Interaction Factor
Fed vs. not fed worms	Scheirer-Ray-Hare Test	Concentration (4 + 1 Control)	Feeding	Concentration Feeding
$CuSO_4$ vs. CuSO4HA	Scheirer-Ray-Hare Test	Concentration (2 + 1 Control)	HA	Concentration HA
$ZnSO_4$ vs. ZnSO4HA	2-way ANOVA	Concentration (2 + 1 Control)	HA	Concentration HA
NP vs. NPHA	Scheirer-Ray-Hare Test	Concentration (4 + 1 Control)	HA	Concentration HA
Bulk vs. BulkHA	Scheirer-Ray-Hare Test	Concentration (4 + 1 Control)	HA	Concentration HA
NP vs. Bulk	Scheirer-Ray-Hare Test	Concentration (4 + 1 Control)	Particle type	Concentration Particle type
NPHA vs. BulkHA	Scheirer-Ray-Hare Test	Concentration (4 + 1 Control)	Particle type	Concentration Particle type

3.3.2 Pilot study: Fed vs. not fed worms

Figure 3.2 shows the behavioural response of worms that were exposed to solutions of varying concentrations of zinc oxide NPs (from 0 – 10mg/L). These worms were either fed at day 7 of their synchronisation period or not fed at all during the synchronisation period. Data were found not to comply with parametric test requirements using a Kolmogorov-Smirnov test ($Z = 1.40$; $P < 0.05$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of feeding worms at day 7 of synchronisation was investigated using a Scheirer-Ray-Hare test. When fed worms were compared to not fed worms across a range of concentrations, the

concentration of NP ($P < 0.001$) and feeding ($P < 0.001$) and the interaction between feeding and concentration were found to be significant ($P < 0.01$) (Table 3.3). Mann Whitney U tests were performed to investigate where these significances lay. Bonferroni corrections were applied to account for the use of multiple tests.

Table 3.3: Statistical results obtained from the Scheirer-Ray-Hare test: influence of feeding and on NP exposure results

Factor	**F**	**SS**	**SS/MStotal**	**d.f.**	**p value**
Concentration	29.39	34171.30	40.60	4	0.001
Feeding	33.45	9721.96	11.55	1	0.001
Conc * feeding	11.42	13274.14	15.77	4	0.01

Effects of NPs on worms that were fed:

The behaviour of worms exposed to 5 and 10mg/L ZnO NPs was significantly inhibited when compared to the control worms ($P < 0.001$). The behaviour of the controls were not found to be significantly different from 1.25 and 2.5mg/L ZnO NPs, however the statistical method is highly conservative and since they are close to significance, this would warrant further consideration. The behavioural response of worms exposed to 1.25, 2.5 and 5mg/L ZnO NPs was not significantly different from each other but were significantly less inhibited than the response of worms exposed to 10mg/L ZnO NPs ($P < 0.003$).

Effects of NPs on worms that were not fed:

For worms that were not fed the control (EPA HW medium alone) was found to have a significantly less inhibited response than all other concentrations ($P < 0.001$). Worms that were not fed and exposed to 1.25mg/L ZnO NPs had a significantly greater inhibition of behavioural response that 2.5 and 10mg/L ZnO NPs ($P < 0.002$) and 2.5, 5 and 10mg/L ZnO NPs were not found to be significantly different from each other.

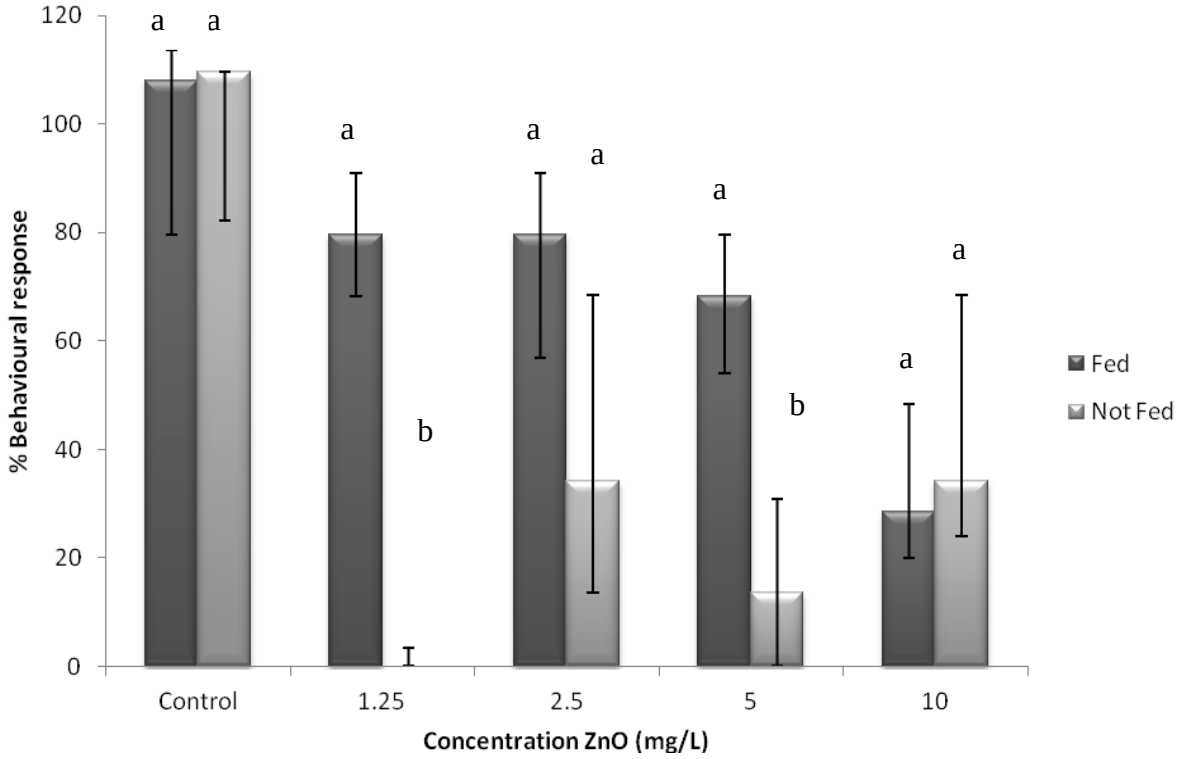

Figure 3.2: Behavioural response (i.e. the number of turns of the worm in response to a tactile stimulus) of worms exposed to various concentrations of ZnO nanoparticles. Worms were fed at day 7 of their synchronisation period and not fed for the 12 day synchronisation period. (Data represents medians (interquartile range), n = 1; comparison made on graph are between each concentration in fed vs. non fed worms – a shared letter between 2 columns indicates no significant difference).

Influence of food on effects of NPs:

The interaction factor was found to be significant ($P < 0.01$) and so the effects of the two factors (feeding and NP concentration) were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to other factor (Dytham, 2011). When comparing fed and not fed worms across each concentration, the controls (EPA HW medium alone) worms and worms exposed to 2.5mg/L and 10mg/L ZnO NPs were not found to be significantly different from each other. Worms exposed to 1.25 and 5mg/L ZnO NPs (fed and not fed) were found to be significantly different from each other ($P < 0.001$) with the fed worms having a less inhibited behavioural response than worms that were not fed. This study suggested that the fed worms were more likely to turn in response to the head flick assay than worms

that were not fed. It was also noted that worms in the fed study had better colouring (i.e. the worms that were not fed were far paler than fed worms) and were more active than non fed worms.

3.3.3 Positive Control Studies

The $CuSO_4$ and $CuSO_4$ with HA data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms (Figure 3.3). Data were normalised to the test mean and outliers were removed using the modified Thompson Tau technique (http:// www.mne.psu.edu/me345/Lectures/ Outliers.pdf, 10/08/2012). Outliers were removed as they are data points that lie outside the usual range of the data and can seriously affect the results of analyses (Quinn and Keogh, 2002). Data were found to be not normally distributed using a Kolmogorov-Smirnov test ($Z = 2.319$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of $CuSO_4$ and $CuSO_4$ with 5mg/L HA was investigated using a Scheirer-Ray-Hare test. The concentration ($F = 31.921$, d.f. = 2, $P < 0.001$) was found to have a significant effect on the behaviour of the worms. As the concentration increased the behaviour of the worms was more inhibited. The addition of HA and the interaction between HA and concentration were not found to be significant factors (Table 3.4).

Table 3.4: Statistical results obtained from the Scheirer-Ray-Hare test: influence of HA on the effect of $CuSO_4$.

Factor	F	SS	SS/MStotal	d.f.	p value
Concentration	31.92	108794.37	46.53	2	0.001
HA	0.001	0.906	0.001	1	0.98
Conc * HA	1.30	4424.22	1.89	2	0.39

Mann Whitney U tests were performed to investigate where the significant differences in concentration lay. Bonferroni corrections were applied to account for the use of multiple tests. No significant differences were found between each control group. The behavioural response of the worms in the control group (EPA HW medium alone) was significantly less inhibited than the response of worms exposed to 0.2µM $CuSO_4$ ($P < 0.01$) and 0.4µM $CuSO_4$ ($P < 0.001$). The control with 5mg/L HA worms also had a

significantly less inhibited behavioural response than those exposed to 0.2µM $CuSO_4$ with HA ($P < 0.001$) and 0.4µM $CuSO_4$ with HA ($P < 0.001$).

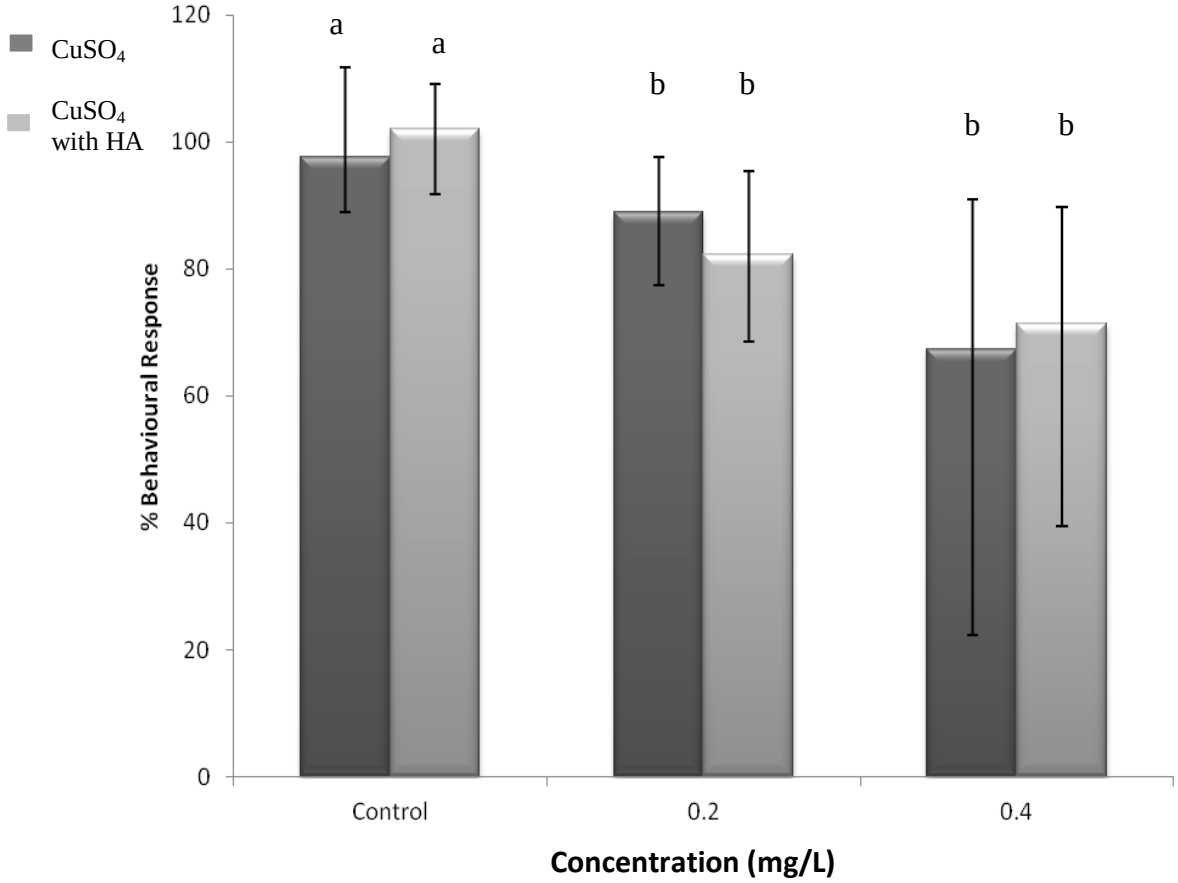

Figure 3.3: Percentage behavioural response of worms exposed to 0.2 and 0.4µM concentrations of $CuSO_4$ and $CuSO_4$ with 5mg/L HA. (Data represents medians with interquartile range, n = 3; comparison made on graph are between each concentration in $CuSO_4$ and each concentration of $CuSO_4$ with HA exposed worms – a shared letter between 2 columns indicates no significant difference. A comparison between concentration with and without HA are not made on the graph as the addition of HA was not found to be significant).

The $ZnSO_4$ and $ZnSO_4$ with HA data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms (Figure 3.4). Data were normalised to the test mean and outliers were removed using the

modified Thompson Tau technique. Data was found to be parametric using a Kolmogorov-Smirnov test ($Z = 1.154$; $P > 0.05$). The effect of $ZnSO_4$ and $ZnSO_4$ with 5mg/L HA was investigated using a 2-way ANOVA with post hoc Tukey tests. The concentration ($F = 9.481$, $P < 0.001$), the addition of HA ($F = 6.788$, $P < 0.01$) and the interaction factor ($F = 4.646$, $P < 0.05$) were all seen to have a significant effect on the behaviour of the worms (Table 3.5).

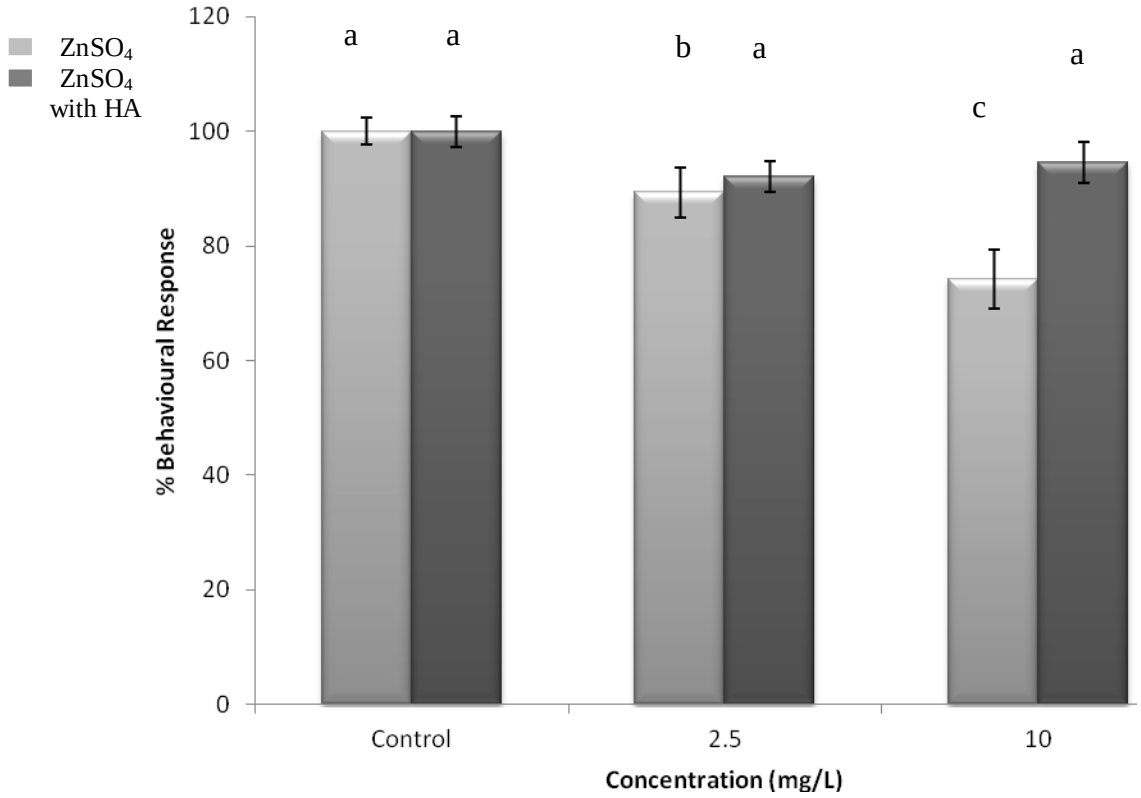

Figure 3.4: Histogram showing the percentage behavioural response of worms exposed to 2.5mg/L and 10mg/L of $ZnSO_4$ and $ZnSO_4$ with 5mg/L HA (data represents medians with interquartile range, n = 3; comparison made on graph are between each concentration and between each concentration and its corresponding HA concentration – a shared letter between 2 columns indicates no significant difference).

Table 3.5: Statistical results obtained from the Scheirer-Ray-Hare test: influence of HA on the effect of $ZnSO_4$.

Factor	**F**	**SS**	**Mean Square**	**d.f.**	**p value**
Concentration	9.48	7151.11	3575.56	2	0.001
HA	6.79	2559.84	2559.84	1	0.01
Conc * HA	4.65	3504.27	1752.13	2	0.01

The behaviour of the worms exposed to 10mg/L $ZnSO_4$ was significantly more inhibited when compared to the control group ($P < 0.001$). The behaviour of worms exposed to 10mg/L $ZnSO_4$ was also significantly more inhibited than those exposed to 2.5mg/L $ZnSO_4$ ($P < 0.05$). The addition of HA improved the behaviour of the worms but only at the highest concentration of 10mg/L $ZnSO_4$ ($P < 0.01$). The interaction factor indicated that the independent variables of concentration and HA both influenced each other and the dependent variable (behavioural response).

3.3.4 Nanoparticle and bulk studies, with and without HA

Effects of NPs and NPs with HA on worms after 96 hours:

The behavioural response data of worms exposed to ZnO NPs and NPs with HA were normalised to the test mean and outliers have been removed using the modified Thompson Tau technique. Data were found to not to be normally distributed using a Kolmogorov-Smirnov test ($Z = 1.804$; $P < 0.01$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of NP concentration and the addition of 5mg/L HA were investigated using a Scheirer-Ray-Hare test. The concentration of NPs was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 6.786$, d.f. = 4, $P < 0.001$). The addition of 5mg/L HA had a significant positive effect on the behaviour of the worms compared to the worms that were treated with particles only ($F = 72.347$, d.f. = 1, $P < 0.001$). The interaction factor between concentration and the addition of HA was also found to be significant ($F = 7.421$, d.f. = 4, $P < 0.001$) (Table 3.6). The significant interaction factor indicated that the concentration and HA variables were dependent on each other as well as the independent behavioural response factor.

Table 3.6: Statistical results obtained from the Scheirer-Ray-Hare test: influence of HA on the effect of ZnO NPs.

Factor	F	SS	SS/MStotal	d.f.	p value
Concentration	6.79	536502.13	22.04	4	0.001
HA	72.35	1429892.37	58.74	1	0.001
HA*Conc	7.42	586661.26	24.10	4	0.001

Since significant differences were founds for all factors, Mann Whitney U tests were performed to investigate where these significances lay. Bonferroni corrections were applied to account for the use of multiple tests. The control in the NP study (EPA HW medium alone) was found to be significantly different from all other ZnO NP concentrations ($P < 0.001$) indicating that worms which were not exposed to NPs had a significantly less inhibited behavioural response that worms exposed to ZnO NPs for 96 hours. Worms exposed to 1.25mg/L, 2.5mg/L, 5mg/L ZnO NPs were not significantly different from each other but 1.25mg/L and 2.5mg/L ZnO NPs were significantly different from 10mg/L ZnO NPs ($P < 0.01$). Worms exposed to 1.25mg/L and 2.5mg/L ZnO NPs had a significantly less inhibited behavioural response than worms exposed to 10mg/L ZnO NPs. There was no significant difference observed in the behaviour of worms exposed to the control with HA and 1.25, 2.5, 5 and 10mg/L ZnO NP with 5mg/L HA. All worms exposed to concentrations of NPs were found to have significantly greater inhibition of behaviour than worms which were exposed to NPs mixed with 5mg/L HA ($P < 0.001$) (Figure 3.5).

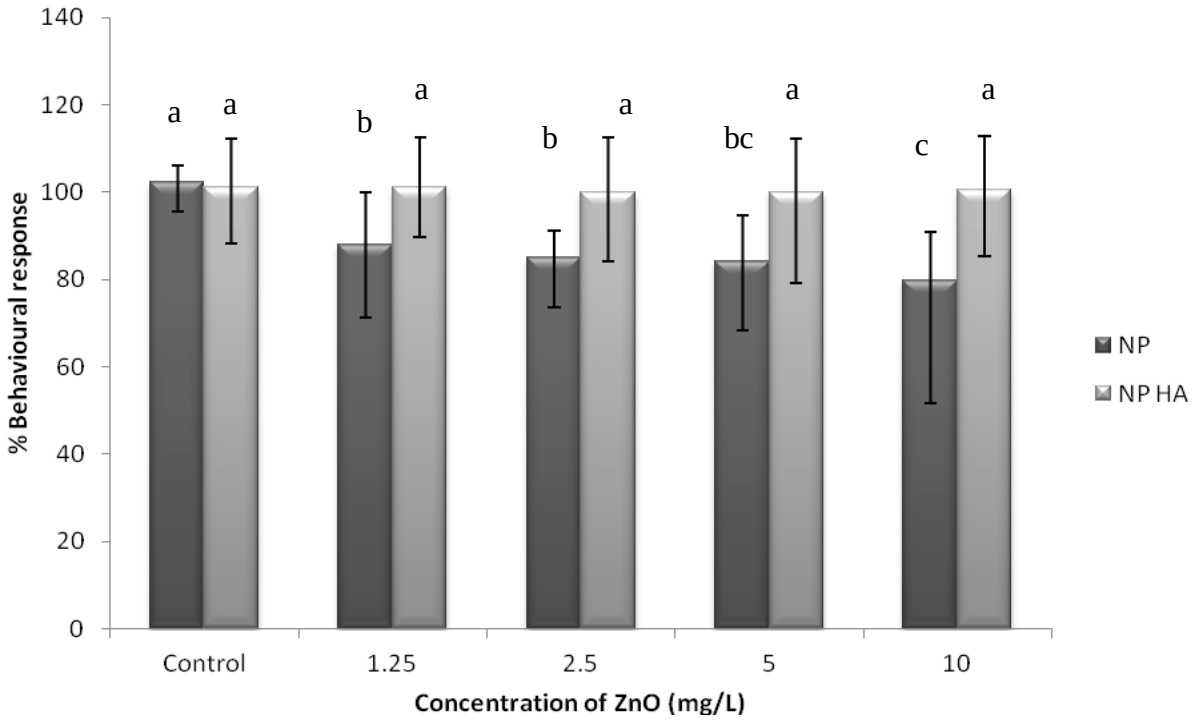

Figure 3.5: Histogram showing the percentage behavioural response of worms (stimulated with a touch stimulus) exposed to 1.25, 2.5, 5 and 10mg/L of ZnO NPs and ZnO NPs with 5mg/L HA. (Data represent medians with interquartile range, n = 6; comparison made on graph are between each and between each concentration and its corresponding HA concentration - a shared letter indicates no significant difference).

Effects of bulk particles and bulk particles with HA on worms after 96 hours:

The behavioural response data of worms exposed to bulk particles and bulk particles with HA (Figure 3.6) were normalised to the test mean and outliers have been removed using the modified Thompson Tau technique. Data were found to be not normally distributed using a Kolmogorov-Smirnov test ($Z = 2.501$; $P < 0.01$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of bulk particle concentration and the addition of 5mg/L HA was investigated using a Scheirer-Ray-Hare test. The concentration of bulk particles was found to have no significant effect on the behaviour of the worms as the concentration increased. The addition of 5mg/L HA had a significant effect on the behaviour of the worms compared to the worms that were treated with particles only ($F = 26.749$, d.f. = 1, $P < 0.001$). The interaction factor between concentration and the addition of HA was also found to be significant ($F = 3.819$, d.f. = 4, $P < 0.01$) (Table 3.7). The significant

interaction factor indicated that the concentration and HA variables were dependent on each other as well as the independent behavioural response factor.

Table 3.7: Statistical results obtained from the Scheirer-Ray-Hare test: influence of HA on the effect of ZnO bulk particles.

Factor	F	SS	SS/MStotal	d.f.	p value
Concentration	2.27	80085.54	8.10	4	0.09
HA	26.75	235923.23	23.86	1	0.001
Conc * ha	3.82	134747.03	13.63	4	0.01

Mann Whitney U tests were performed to investigate where these significances lay. Bonferroni corrections were applied to account for the use of multiple tests. Worms exposed to 5mg/L bulk particles with 5mg/L HA were found to have a significantly inhibited behavioural response when compared to the control with HA group ($P < 0.01$). When comparing the response of the worms exposed to bulk particles and those exposed to bulk with 5mg/L HA it was found that only 5mg/L ZnO bulk particle was significantly different from their HA counterpart ($P < 0.001$) indicating that the worms had the same response to bulk particles and bulk particles with 5mg/L except at 5mg/L ZnO bulk particles.

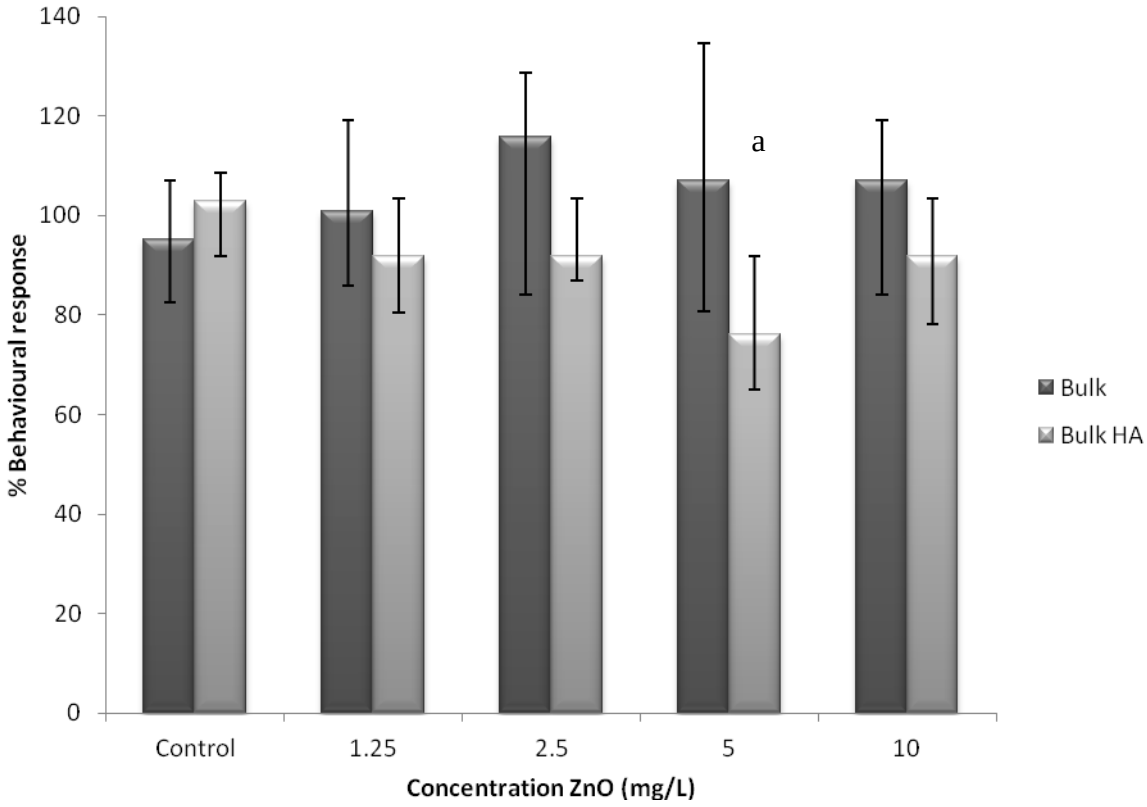

Figure 3.6: Percentage behavioural response of worms (stimulated with a touch stimulus) exposed to various concentrations of ZnO bulk particles and bulk particles with 5mg/L HA. . (Data represent medians with interquartile range, n = 4; comparison made on graph are between each and between each concentration and its corresponding HA concentration - a letter indicates significant difference).

Effects of ZnO NP vs bulk particles (without HA) on the worms:

The NP and bulk data (without HA) were compared to each other to investigate whether there were any significant differences in the behaviour of the worms exposed to either particle type (Figure 3.7). Data were normalised to the test mean and outliers have been removed using the modified Thompson Tau technique. Data were found to not to be normally distributed using a Kolmogorov-Smirnov test ($Z = 1.407$; $P < 0.05$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of NP concentration and bulk concentration was investigated using a Scheirer-Ray-Hare test. The concentration ($F = 3.650$, d.f. = 4, $P < 0.05$), particle type ($F = 78.112$, d.f. =1, $P < 0.001$) and the interaction between concentration

and particle type (F = 7.902, d.f. = 4, P < 0.001) were all found to be significant (Table 3.8).

Table 3.8: Statistical results obtained from the Scheirer-Ray-Hare test: assessing the effect of ZnO size on worm behaviour.

Factor	F	SS	SS/MStotal	d.f.	p value
Concentration	3.65	190158.70	11.34	4	0.022
Particle	78.11	1017282.52	60.69	1	0.001
Conc*particle	7.90	411632.82	24.56	4	0.001

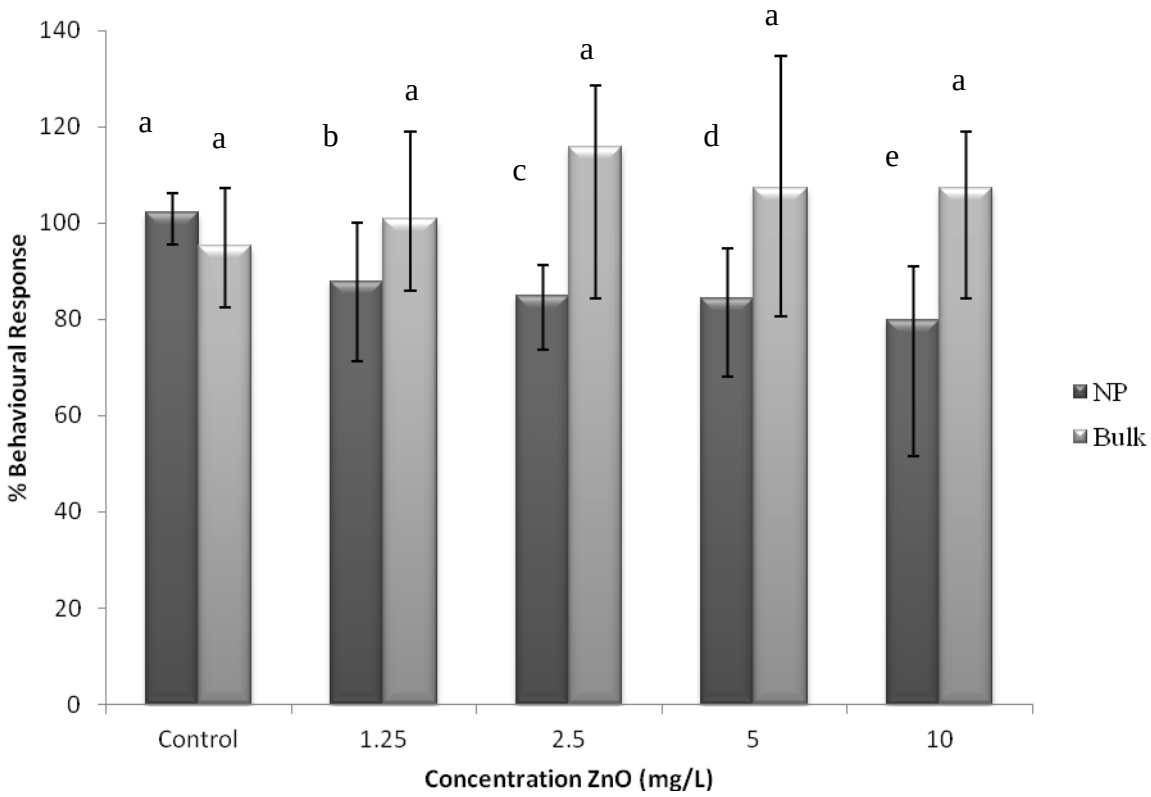

Figure 3.7: Percentage behavioural response of worms (stimulated with a touch stimulus) exposed to various concentrations of ZnO NPs and ZnO bulk particles. (Data represent medians with interquartile range, n = 4; comparison made on graph are between each and between each concentration and its corresponding HA concentration - a shared letter indicates no significant difference).

Mann Whitney U tests were performed to investigate where these significances lay. Bonferroni corrections were applied to account for the use of multiple tests. The concentration of nanoparticles was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 6.786$, d.f. = 4, $P < 0.001$). The concentration of bulk particles was found to have no significant effect on the behaviour of the worms as the concentration increased. There was no significant difference found between the control group of the NP study and the control group of the bulk particle study. Worms in 1.25mg/L, 2.5mg/L, 5mg/L and 10mg/L NP were found to have a significantly inhibited behavioural response when compared to their bulk counterparts ($P < 0.001$). The interaction factor was found to be significant ($P < 0.001$). The significant interaction factor indicated that the concentration and particle type variables were dependent on each other as well as the independent behavioural response factor.

Effects of ZnO NP vs bulk particles (with HA) on the worms:

The NP with HA and bulk with HA data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms exposed to either particle type with HA (Figure 3.8). Data were normalised to the test mean and outliers have been removed using the modified Thompson Tau technique. Data were found to be not normally distributed using a Kolmogorov-Smirnov test ($Z = 1.551$; $P < 0.05$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of NP with 5mg/L HA concentration and bulk with 5mg/L HA concentration was investigated using a Scheirer-Ray-Hare test. The concentration ($F = 4.371$, d.f. = 4, $P < 0.01$), particle type ($F = 13.818$, d.f. =1, $P < 0.001$) and the interaction between concentration and particle type ($F = 2.859$, d.f. = 4, $P < 0.05$) were all found to be significant (Table 3.9). The significant interaction factor indicated that the concentration and particle type with HA variables were dependent on each other as well as the independent behavioural response factor.

Table 3.9: Statistical results obtained from the Scheirer-Ray-Hare test: influence of HA on the effect of ZnO particles (NP and bulk).

Factor	F	SS	SS/MStotal	d.f.	p value
Concentration	4.37	259658.82	16.35	4	0.01
Particle	13.82	205230.54	12.93	1	0.001
Conc * particle	2.86	169833.30	10.70	4	0.03

Mann Whitney U tests were performed to investigate where these significances lay. Bonferroni corrections were applied to account for the use of multiple tests. There were no significant differences across the NP with HA concentrations but there were significant differences seen across the bulk with HA concentrations. Worms exposed to 5mg/L bulk with HA were found to have a significantly lower behavioural response when compared to the bulk with HA control worms ($P < 0.05$). When comparing worms exposed to NP with HA and worms exposed to bulk with HA significant differences were found. Worms exposed to 5mg/L ($P < 0.001$) NP with HA were found to have a significantly less inhibited response than worms exposed 5mg/L bulk with HA.

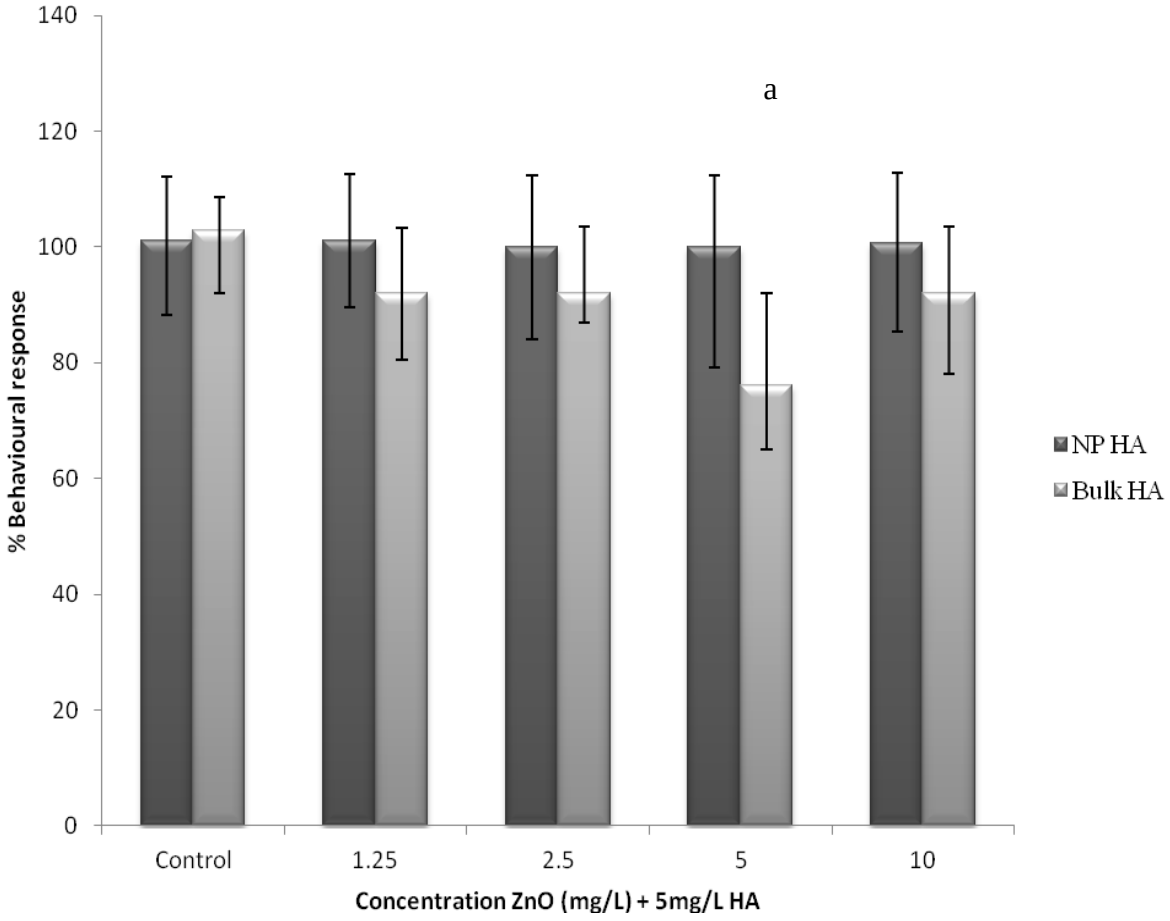

Figure 3.8: Percentage behavioural response of worms (stimulated with a touch stimulus) exposed to various concentrations of ZnO NPs with 5mg/L HA and ZnO bulk particles with 5m g/L HA. (Data represent medians with interquartile range, n = 4; comparison made on graph are between each and between each concentration and its corresponding HA concentration - a letter indicates significant difference).

3.4 Discussion

3.4.1 Feeding worms during physiological synchronisation

The worms were physiologically synchronised prior to toxicity testing according to a protocol from the OECD 225 guideline (2007). Within this protocol it is recommended that worms be fed as soon as they exhibit burying behaviour, if kept on sediment, or at day seven of synchronisation if kept in artificial paper "sediment". Leppänen and Kukkonen (1998) stated that the worms cease to ingest during division into fragments. Previous studies conducted within our research group and in the literature (e.g. Liebig *et*

al., 2005), however, were conducted without feeding the worms during the synchronisation period. In order to test whether this would affect the behaviour of the worms during testing an investigation was performed with and without feeding of the worms at day seven. Feeding the worms at this point in their physiological synchronisation resulted in worms that were more likely to respond to a tactile stimulus during testing. Prior to synchronisation worms were kept in culture and fed weekly. A lack of food during synchronisation may be viewed as an adverse condition and so can affect the growth of *L. variegatus*. A lack of sufficient growth and healthy status of the worms would introduce a further variable into the NP toxicity tests and would also be less environmentally relevant. The interaction factor between NP concentration and feeding was found to be significant and therefore not additive. If two variables interact the relationship between each of the interacting variables and the dependent variable depends on the value of the other interacting variable, i.e. the ability of the worm to respond to a tactile stimulus was affected by both the NP concentration and feeding during synchronisation. It has been suggested that growth and reproduction in *L.* variegatus can be affected by adverse conditions (Leppänen and Kukkonen, 1998). As a result of this study, the null hypotheses that feeding the worms at day seven would not affect the behaviour of the worms and would have no effect on the behaviour of the worms during exposure to NPs were rejected and all subsequent synchronisations of worms in this thesis (i.e. all water only studies, all oxidative stress studies, all sediment and paper sediment studies, all depuration studies and all histological studies) were conducted with feeding on day seven.

3.4.2 Positive control tests

A positive control is designed to show that a test system is capable of detecting/determining the end point in question, using a perturbation distinct from that used in the study (Glass, 2007). In this case copper sulphate and zinc sulphate were used as positive controls. The positive controls both had a dose dependent significant negative effect on the behaviour of the worms after the 96 hour testing period, indicating that the experimental protocol was valid.

3.4.3 Acute ZnO NP toxicity vs. acute ZnO bulk particle toxicity

In this study the effects of particle type and the addition of organic matter to the particle dispersion on the behavioural response of *L. variegatus* were investigated.

Comparison of the effects of ZnO NP and bulk particles (without HA) on the worms:

The null hypothesis that there is no significant difference in the behaviour of worms exposed to NPs and those exposed to bulk particles was rejected as significant differences in behavioural response was seen across all concentrations up to 10mg/L in the NP verses bulk. The interaction factor between the concentration of the particles and the particle type was also found to be significant, indicating that since these two variables interact the relationship between concentration and particle type and the dependent variable, behavioural response, depends on the value of the other interacting variable.

The surface area of the NPs was found to be greater than that of the bulk particles (BET study, Chapter 2, Section 2.3.2) and this may explain why the NPs were found to have a toxic effect when the bulk did not. As mentioned in Chapter 2 (Section 2.4.2), the greater the surface area of a particle, the greater the reactivity of that particle and in turn the greater the potential for that particle to have an increased toxicity (e.g. Pal *et al*., 2007; Navarro *et al*., 2008; Li *et al*., 2006).

The solubility of the particles may also have played a role in the difference in observed toxicity. The solubility of the NPs (0.9 ± 0.15 (SE) mg/L) was found to be significantly greater than that of the bulk particles (0.6 ± 0.13 (SE) mg/L) in dialysis experiments (Chapter 2, Section 2.3.2). This would mean that the worms were exposed to more Zn ions in the NP exposures compared to the bulk particle exposures, giving rise to potential ionic toxicity. However, they may also have been exposed to insoluble NP/aggregates as the solubility equilibrium was reached at 0.9 ± 0.15 (SE) mg/L and the exposure concentrations in this experiment ranged from 1.25 to 10mg/L ZnO. Studies, which have used crustaceans (Blinova *et al*., 2010), protozoa (Blinova *et al*., 2010) and algae (Franklin *et al*., 2007; Aruoja *et al*., 2009), have stated that the toxicity observed in their studies was down to ions rather than NPs. Some studies, however, in human toxicology (cell lines; Xia *et al*., 2008), mammalian toxicology (adult mice; Wang *et al*., 2008) and ecotoxicology (crustaceans and bacteria; Heinlaan *et al*., 2008) have also

shown nano-specific toxicity. Xia *et al*. (2008) stated that the cytotoxicity observed in their cell line study was down to both the dissolution and release of Zn^{2+} in the culture medium as well as the uptake of the particle remnants (i.e. particles that had shed their label) by lysosomes in the cell. This accumulation of Zn in the lysosomes caused a number of issues within the cell, e.g. oxidative stress and cytotoxicity. Generalisations cannot be made to state that ZnO NP toxicity is ultimately solely down to Zn^{2+} ion release (Wong *et al*., 2010) and the dissolution of NPs may result in an additional layer of complexity when examining their potential toxicity (Xia *et al*., 2008).

Finally, the feeding behaviour of *L variegatus* may also have played a role in the difference in observed toxicity between ZnO NPs and bulk particles. *L. variegatus* are known to be "conveyor belt" feeders (Brinkhurst and Jamieson, 1971) and ingest fine particulate matter (Gaskell *et al*., 2007). The agglomerates that the worms may have come in contact with in the bulk study may potentially have been too large for them to ingest. Within the DLS study (Chapter 2, Section 5.3.3) the hydrodynamic diameter of bulk ZnO particles were found to be significantly larger than ZnO NPs.

Comparison of the effects of ZnO NP and bulk particles (with HA) on the worms:

In this study the effect of particle type with the addition of organic matter to the particle dispersion on the behavioural response of *L. variegatus* was investigated. The null hypothesis that there is no significant difference in the behaviour of worms exposed to NPs with 5mg/L HA and those exposed to bulk particles with 5mg/L HA was rejected as a significantly greater inhibition of behavioural response was observed at 5mg/L bulk with HA compared to 5mg/L NP with HA. The interaction factor between the concentration of the particles with HA and the particle type was also found to be significant, indicating that since these two variables interact the relationship between concentration with HA and particle type and the dependent variable, behavioural response, depends on the value of the other interacting variable.

The addition of humic acid mitigated the negative effect of NPs to the point where NPs mixed with 5mg/L HA had no effect on the behaviour of the worms, allowing for the rejection of the null hypothesis that the addition of HA had no effect on the toxicity of the particle. TEM images taken of ZnO NPs dispersed with 5mg/L HA (Chapter 2, Section 2.3.2) indicate that the particles agglomerated and were also

surrounded by what appeared to be an organic matrix. This matrix may have been what increased the stability of the dispersion (indicated by DLS data, Chapter 2, Section 2.3.1) and kept the agglomerates in suspension throughout the exposure. Other studies have noted that humic acids increase the stability of NP suspensions (Chen and Elimelech, 2007; Handy *et al.*, 2008; Zhang *et al*., 2009). It is also possible that the HA acted as a chelating agent. HA is a natural chelating polymer and is often used as a sink for many multivalent metal ions because of its widespread availability and ability to bind metal ions (Yaghmour *et al*., 2007).As the worms sit at the base of the vial there is potential for them to have come into less contact with the particles compared to NP exposures alone.

The solubility of ZnO NPs in EPA HW medium and EPA HW with HA medium was not found to be significantly different so the worms in the HA exposures had the potential to come into contact with the same amount of Zn ions but potentially had less contact with ZnO NPs and agglomerates. A study conducted by Gao *et al*. (2012) using Ag NPs stated that the addition of organic matter did not affect solubility and also that toxicity linearly decreased as the levels of organic matter increased in the exposure solution. However, at 5mg/L ZnO bulk with 5mg/L HA there was a significant negative effect on the behaviour of the worms and so the null hypotheses that the addition of HA (across increasing concentrations of bulk ZnO up to 10mg/L) had no effect on the toxicity of the bulk particles was rejected. When examining the DLS data, HA does not appear to affect the hydrodynamic diameter of the bulk particles however their stability was increased with the addition of 5mg/L HA. It would be expected that since HA should have increased dispersion and kept the bulk particles in suspension, the worms would have come into less contact with the particles. However, it is possible that large agglomerates may have formed and deposited onto the area where the worm was placed. The TEM images produced at FENAC did appear to show agglomerates that looked different in EPA HW medium and EPA HW medium with HA, suggesting that there was little effect of the HA on agglomeration of ZnO NPs. Statistics performed did not show that there was a significant difference however the data may not have been sufficient to perform robust testing.

An important consideration within these studies is the possibility bias from the observer. In order to prevent such bias blind controls could be used and should be considered for future studies.

3.5 Summary of this study

The behavioural response of *L. variegatus* after exposure to ZnO NPs and bulk particles was investigated in this study. ZnO NPs (1.25 – 10mg/L) were found to have a significant negative effect on the behaviour of the worms after a 96 hour exposure however this effect is mitigated by the addition of 5mg/L HA. ZnO bulk particles (with and without 5mg/L HA) were not found to have any effect on the behaviour of the worms except at 5mg/L ZnO bulk particle with 5mg/L HA. The possible reasons for the difference in toxicity between NPs and bulk particles are the feeding behaviour of the worms, the size difference between the particle, the dissolution of the particles and also the stability of the particles in suspension. These studies suggest that although there may have been ionic toxicity there was also a nano-specific effect observed. The aims of the study were met and this sub lethal bioassay was found to be a successful endpoint for investigating the toxicity of NPs in *L. variegatus*.

Chapter 4 Acute Toxicity Testing: Investigation of the potential for ZnO nanoparticles to induce oxidative stress in *Lumbriculus variegatus*.

4.1 Introduction

Due to the physicochemical characteristics of nanoparticles they are expected to have a greater potential for toxicity via mechanisms such as oxidative stress. NPs that are chemically unstable in media can produce reactive oxygen species (ROS) which can lead to oxidative stress in the presence of organisms (Houdy *et al*., 2011). A number of studies have indicated that nanoparticles do induce oxidative stress and damage in both human models (e.g. epithelial, lung and kidney cells) and environmental models (e.g. fish, molluscs and annelids). There is evidence to suggest that accumulated damage from oxidative stress contributes to numerous diseases, such as cancer, artherosclerosis, neurodegenerative diseases and rheumatoid arthritis (Aruoma, 1998) and so is an important biomarker for investigation.

4.1.1 Oxidative stress and antioxidant defences

As mentioned in Chapter 1, oxidative stress occurs when there is an imbalance between oxidants and antioxidants in a cell (Sies 1985, 1986, 1991) and can potentially lead to damage. Oxidants are normal products of aerobic metabolism but can be produced at elevated levels under stressed conditions (Sies, 1997). Oxidative stress can be induced by reactive oxygen species and free radicals. Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen. Molecular oxygen can be evolved into an array of reactive metabolites (e.g. hydroxyl radicals, superoxide radicals, hydrogen peroxide and singlet oxygens) by reactions which can cause extensive oxidative damage (Sohal and Weindruch, 1996). It is estimated that ~2-3% of oxygen consumed by aerobic cells is converted to O_2^- and H_2O_2 (Chance *et al*., 1979). Free radicals can also cause oxidative stress. A free radical is any species that is able to exist independently and that contains paired and unpaired electrons (Halliwell, 2006). A state of oxidative stress exists in cells because of an imbalance between oxidants and

antioxidants (Sohal and Weindruch, 1996) and is brought about as mentioned by exposure of the cell to reactive oxygen intermediates, such as superoxide anions ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\cdot$). These intermediates if uncontrolled or excessive in amount can lead to oxidative stress including damage to proteins, nucleic acids and cell membranes (Storz and Imlay, 1999). Generally, ROS have been proposed to work in 2 ways to damage the function of the cell: (i) by interacting with a large variety of biomolecules which can lead to tissue necrosis and the potential death of the organism and (ii) by interfering with the expression of genes and signal transduction pathways (Apel and Heribert, 2004).

In order to protect themselves from oxidative stress cells possess a number of antioxidant strategies, most of which are expressed at low levels during normal cell life cycles (Storz *et al.*, 1999). Halliwell and Gutteridge (1989) stated that an antioxidant is "any substance that when present at low concentrations compared with that of an oxidisable substrate significantly delays or inhibits oxidation of that substrate". Antioxidants can act at various levels of the oxidative stress sequence and they may have multiple mechanisms of action (Halliwell and Gutteridge, 1989). Antioxidants can be broadly described in two groups: i) the enzymatic defences and ii) the non-enzymatic defences. The enzymatic defences maintain levels of ROS by reducing the excess ROS to H_2O and the non-enzymatic defences are free radical scavengers (Krishnan and Kodrík; In Farooqui and Farooqui, 2012). Examples of enzymatic antioxidant defences include superoxide dismustase (SOD), catalase (CAT) and glutathione-S-transferase (GST). SOD is responsible for the conversion of superoxide into H_2O_2 and oxygen (Halliwell and Gutteridge, 2007). CAT catalyses the decomposition of H_2O_2 into H_2O and O_2 (Abdel-Mageed *et al.*, 2012) and GST catalyses the reaction of the major low molecular mass thiol, glutathione (GSH) (Sies, 1997), which will be discussed further below.

An example of a non-enzymatic defence is glutathione (GSH), an important free radical scavenger. GSH is oxidised by ROS to form oxidized GSSG. GSH exists ubiquitously in living systems and plays a crucial role in cellular defence against oxidative stress in tissues and cells (Kosower and Kosower, 1978; Kidd, 1997; Sen, 1997). As electrons are lost from GSH the molecule becomes oxidized and two such molecules link together to form GSSG via a disulphide bridge. This linkage is reversible upon re-reduction. GSH recycling is catalysed by glutathione disulfide

reductase using NADPH to reconvert GSSG to 2 GSH (Asada and Takahashi, 1987; Meister, 1994) however when NADPH becomes limited GSSG cannot be converted back to GSH and GSH is depleted. A dynamic balance is maintained between GSH synthesis, its recycling from GSSG and it utilisation (Kidd, 1997). The dynamic balance between GSH and GSSG is important in cellular signal transduction, gene regulation, redox regulation and biochemical homeostasis (Senft *et al*., 2000). The intracellular depletion of GSH can lead to cell death and its clinical relevance has been researched for a number of years (Cook and Sherlock, 1965). Factors which can induce oxidative stress via the depletion of GSH include UV and other radiation (Cai *et al*., 2000), viruses (Kidd, 1997), environmental toxins, chemicals, heavy metals (Kidd, 1997), inflammation due to injury (Luo *et al*., 1998; Spies *et al*., 1994) and dietary deficiencies of GSH precursors and enzyme cofactors (Whitcomb and Block, 1994).

4.1.2 Oxidative stress and nanoparticles

As mentioned previously, a number of studies have shown that NPs can cause oxidative stress in both human (e.g. epithelial cells, lung cells and kidney cells) and environmental models (e.g. freshwater and marine fish, freshwater and marine invertebrates and terrestrial invertebrates). One of the first papers was by Stone *et al*. (1998) which used carbon black ultrafine particles in lung epithelial cell line (A549) which depleted glutathione. TiO_2 NPs (e.g. used in domestic and cosmetic applications) were found to compromise the antioxidant defence system of human amnion epithelial (WISH) cells via a depletion GSH and CAT activity and to increase intracellular ROS production causing oxidative stress (Saquib *et al*., 2012). CeO_2 NPs (e.g. used as a catalyst) were also found to induce oxidative stress, via GSH depletion and increased ROS production, in human lung (BEAS-2B) cells (Park *et al.*, 2008). Kim *et al.* (2011) also found that oxidative stress was induced via oxidative DNA damage in BEAS-2B cells by Ag NPs (e.g. medical applications) as ROS production was increased in the cells after exposure to Ag NPs. ZnO, CdS and SiO_2 NPs were found to cause oxidative stress in human kidney cells via a depletion of GSH. ZnO and CdS NPs were found to increase ROS production and cause oxidative stress in IP15 and HK-2 cells (Pujalté *et* al., 2011) and SiO_2 NPs were found to cause a reduction in GSH content and an increase in intracellular ROS in HEK293 kidney cells (Wang *et al*., 2009).

Ag NPs have been shown to cause oxidative stress in Tilapia (*Oreochromis mossambicus*) by compromising the antioxidant defence system (inhibition of CAT activity), lipid peroxidation (LPO) and a heavy accumulation of free radicals (Govindasamy and Rahuman, 2012), in adult zebra fish (*Danio rerio*) via LPO and an increase in GSH (Choi *et al.*, 2010) and embryonic medaka (*Oryzias latipes*) via a depletion in GSH (Wu and Zhou, 2012). They have also been shown to cause oxidative stress in terrestrial invertebrates *Drosophila melanogaster* (Ahamed *et al.*, 2010) and *Eiseina fetida* (Tsyusko *et al.*, 2012). Ahamed *et al*. (2010) reported that Ag NPs caused a reduction in GSH and an increase in SOD and CAT activity in the fruit fly and Tsyusko *et al*. (2012) reported that CAT activity was increased in the earthworm after exposure to Ag NPs, which indicated oxidative stress. TiO_2 NPs were shown to cause oxidative stress in marine abalone (*Haliotis diversicolor supertexta*) by decreasing GSH content and increasing SOD activity (Zhu *et al*., 2011) and in nematode worms (*Caenorhabditis elegans*) by increased ROS production (Wu *et al.*, 2012). TiO_2 was also found to cause oxidative stress in water fleas (*Daphnia magna*) by increasing enzyme activity (CAT, glutathione peroxidise (GPX) and GST) (Tae Kim *et al*., 2010). Cu NPs induced oxidative stress in mussels (*Mytilus galloprovincialis*) via LPO, the increase in CAT and SOD activity and by ROS production (Gomes *et al*., 2011) and CuO NPs were found to cause oxidative stress in marine worms (*Scrobicularia plana* and *Hediste diversicolor*) by increasing enzyme activity (CAT and GST) (Buffet *et al*., 2012). Au NPs were shown to cause oxidative stress via LPO in blue mussels (*Mytilus edulis*) with increased production of ROS (Tedesco *et al.*, 2010).

4.1.3 Oxidative stress and ZnO nanoparticles

A number of studies have indicated that ZnO NPs caused oxidative stress in a variety of species. Ali *et al*. (2012) stated that ZnO NPs (32µg/ml) induced oxidative stress in *Littoraria luteola* via a decline of GSH in the digestive gland of the snail and Chandran *et al*. (2005) also indicated that a decline of GSH was observed in the digestive gland and kidney of ZnO NP (1mg/L) treated *Achatina fulica*. Xiong *et al*. (2011) stated that ZnO NPs (4.92mg/L) caused disturbances in the oxidative defences via LPO in the gill, gut and liver tissues of zebra fish. Hao and Chen (2012) stated that (50mg/L) ZnO NPs caused a decrease in enzyme activities and also a decrease in GSH content in carp

(*Cyprinus carpio*) indicating oxidative stress. ZnO has also been shown to cause oxidative stress both *in vitro* and *in vivo* in of human models. *In vitro* human cell line studies (e.g. BEAS 2-B and A549 cells) indicate that the induction of oxidative stress is the most important or likely mechanism underlying ZnO toxicity (Vandebriel & De Jong, 2012). Fukui *et al*. (2012) reported that ZnO NPs caused an oxidative stress response in a rat lung (intratracheal instillation) and in culture (A549 cells) and Sharma *et al*. (2012) reported that ZnO caused LPO which lead to oxidative stress in mouse liver *in vivo*. ZnO NPs have also been shown to cause oxidative stress *in vitro,* for example in human epithelial (BEAS-2B) cells (Huang *et al.*, 2010) and in human colon carcinoma cells (De Beradis *et al.*, 2010) by increased ROS production.

4.1.4 Oxidative stress and L. variegatus

L. variegatus have been used extensively to assess bioaccumulation and toxicity, however less is known about biotransformation of toxic substances, the defences used to detoxify those substances and how this oligochaete is protected from oxidative damage (Wiegand *et al*., 2007). Very few studies have been published to date in this area. Wiegand *et al*. (2007) investigated the oxidative stress induced by paraquat (with and without dissolved organic matter (DOM)) using soluble glutathione S transferase (sGST), catalase (CAT) and peroxidase (POD) markers. They stated that oxidative stress occurred as indicated by increased levels of sGST (with and without DOM), reduced CAT (with DOM), increased CAT (without DOM), but no increase in POD. Cochón *et al*. (2007) also investigated the toxicity of paraquat by looking at LPO, CAT and superoxide dismutase (SOD). They reported that there was no lipid peroxidation, a decrease in CAT (however this was transient and only occurred after 4 hours) and there was no change in SOD. Contardo-Jara and Wiegand (2008) investigated the toxicity of anthropogenically contaminated sediments (pesticides and metals) and atrazine using sGST and CAT markers. They reported that an increase was observed after 4 days in sGST and an increase in CAT only after 1 day. Kristoff *et al*. (2008) used GSH, SOD, CAT and sGST to investigate the oxidative stress potential of azinophos-methyl. They reported that GSH was increased, SOD and CAT were decreased and there was no change in sGST. Finally, Contardo-Jara *et al*. (2009) reported on the oxidative stress induced in the worms by exposure to "Round Up" (herbicide). They reported that

oxidative stress was induced as both sGST and SOD were increased. The findings from these studies are summarised in Table 4.1 below. Only one study investigated GSH as a marker of oxidative stress and all used multiple markers suggesting that multiple markers may give more information that using GSH alone.

Table 4.1: Summary table of results for studies which use *L. variegatus* to investigate oxidative stress (↑ = increase, ↓ = decrease, - = no change, empty = marker not investigated)

Author	**Contaminant/ Marker**	**sGST**	**CAT**	**POD**	**LPO**	**SOD**	**GSH**
Wiegand *et al*., 2007	Paraquat (with DOM)	↑	↓	-			
Wiegand *et al*., 2007	Paraquat (– DOM)	↑	↑	-			
Cochón *et al*., 2007	Paraquat		↓		-	-	
Contardo-Jara *et al*., 2008	Contaminated Sediment + Atrazine	↑	↑				
Kristoff *et al*., 2008	Azinophos-methyl	-	↓			↓	↑
Contardo-Jara *et al*., 2009	Round Up (Herbicide)	↑				↑	

4.1.5 Aims of this study

Antioxidant defences are found widely in aquatic species (Valavanidis *et al*., 2006; Lushchak, 2011). Measurement of the antioxidants can be used as biomarkers for adverse health effects of environmental pollutants (Lemaire *et al*., 1993; Livingstone, 2001). Aquatic organisms can provide models for the investigation of ROS induced damage, cellular response, repair mechanisms and how oxidative stress can lead to

disease (Di Giulio *et al*., 1989; Livingstone *et al*., 1993). The development of sublethal bioassays in *L. variegatus* could provide diagnostic and prognostic early warning tests for monitoring the environmental health of a particular site. The aim of this study was to investigate the effects of ZnO NPs and bulk particles on the level of GSH in the cells of *L. variegatus* in water only acute toxicity tests, over a range of concentrations, with and without Suwannee River humic acid and at various times points. The null hypotheses investigated in this study were that ZnO NPs and bulk particles would have no significant effect on the oxidative stress reaction of *L. variegatus*.

4.2 Methods

Two protocols were used in this study in order to ascertain which was the best method for detecting and quantifying GSH in a sample of worm tissue. The first protocol employed was adapted from Senft *et* al. (2000) which used *o*-Phthalaldehyde (OPT), a fluorescent probe, to indicate the levels of GSH in a sample. OPT is a flurogenic agent which can react with the organic molecule containing primary amino group in the presence of a thiol in an alkaline medium to give a fluorescent product (Roth, 1971; Taylor and Tappel, 1973) that can be detected and specifically quantified. The second protocol used to measure GSH in a sample was a "GSH-Glo^{TM} Glutathione Assay" kit purchased from Promega (U.K.) which was a luminescence assay based on the conversion of a luciferin derivative into luciferin and hence light in the presence of GSH (catalysed by GST) to detect and quantify GSH in a sample.

The investigation of the potential for ZnO NPs and bulk particles to induce oxidative stress in *L. variegatus* was conducted in a number of steps:

1. A pilot study to conclude how many worms were needed per treatment to get an accurate reading on the spectrophotometer with a protocol adapted from Senft *et al*. (2000), (Section 4.2.1);
2. A further pilot with a kit purchased from Promega (Section 4.2.2) in order to ascertain whether this protocol would reduce the time and amount of tissue needed to complete the study;
3. A full study based on the results obtained from the Promega kit used in the pilot study (Section 4.2.3).

4.2.1 Study 1: Pilot study using adapted OPT protocol

This protocol was adapted from Senft *et al*. (2000). The reagents necessary for this study were prepared fresh on the morning of the study. Redox Quenching Buffer (RQB) was prepared by measuring 490ml of deionised water into a glass bottle. Following this, 10ml of 1M HCl, 0.4g EDTA and 0.88g of ascorbic acid were added to the water in a fume cupboard and allowed to dissolve. The solution was stored at 4˚C. A solution of 5% TCA in RQB was prepared by adding 5g of TCA to 100ml RQB buffer. The solution was stored at 4˚C. Buffers were prepared of 1M potassium phosphate pH 7.0 and 0.1M potassium phosphate pH 6.9. For the pH 7.0 buffer 6.8g of KH_2PO_4 was dissolved in 30ml of distilled water and the pH was adjusted to 7.0 with 10N NaOH. The volume of the solution was made up to 50ml and stored at 4˚C. The pH 6.8 buffer was made by dissolving 6.8g of KH_2PO_4 in 200ml of distilled water and the pH was adjusted using 10N NaOH. The volume was then completed to 500ml with distilled water and stored at 4˚C. A 7.5mM NEM in RQB was prepared by dissolving 4.7mg NEM in 5ml of RQB buffer and subsequently stored on ice. A 5mg/ml OPT in methanol was prepared by dissolving 10mg OPT (under a fume hood) in 2ml of methanol. This was then stored on ice and protected from light in a foil covered bijou tube. A fresh preparation of 10mM sodium dithionite in RQB was prepared (for the total GSH plate readings) by dissolving 17.4mg of dithionite in 1ml RQB under a fume cupboard.

Standards were made up from a stock solution of GSH at 0.05M which was prepared by dissolving 15mg of reduced GSH in 1ml of 5% TCA in RQB and was stored on ice. From this stock a 1 in 100 dilution was made i.e. 990µl 5% TCA in RQB and 10µl 0.05M GSH, to give a 0.5mM GSH solution. From this diluted solution 6 further solutions were made of 3.125µM GSH (500 µl 5% TCA in RQB and 500 µl 6.25 µM GSH), 6.25µM GSH (500 µl 5% TCA in GSH and 500 µl 12.5 µM GSH), 12.5µM GSH (500 µl 5% TCA in RQB and 500 µl 25 µM GSH), 25µM GSH (500 µl 5% TCA in RQB and 500 µl 50 µM GSH), 50µM GSH (900 µl 5% TCA in RQB and 100 µl 0.5mM GSH) and 100µM GSH (800µl 5% TCA in RQB and 200µl 0.5mM GSH). A control solution (0µM GSH) was prepared using 1ml 5% TCA in RQB. Negative controls were also prepared. A 25mM solution of GSSG was prepared by dissolving 15mg GSSG in 1ml of 5% TCA in RQB. This was then diluted 1 in 100 to give a

solution of 250µM GSSG. This was further diluted by mixing 400µl of the 250µM solution and 600µl of 5% TCA in RQB.

L. variegatus were taken directly from the culture tanks for the pilot study i.e. they were not physiologically synchronised. Worms were dried as much as possible on unbleached paper towels and weighed. Unexposed worms in batches of 5, 10, 20 and 30 worms were homogenised using a hand held homogenising device in separate eppendorf vials in 250µl of lysis buffer. The eppendorfs were then centrifuged at 4˚C and 15,000 g for 5 minutes and the supernatant was used for the assay.

For reduced GSH readings, a black, flat bottomed 96 well plate was rinsed clean under distilled water prior to adding any reagents. Firstly, 19µl of 5% TCA in RQB was added per well, followed by 10µl of standard or sample. Following this 4µl of RQB was added per well or 7.5mM NEM was added in the well chosen as a back ground control. Next 48µl of 1M KH_2PO_4 pH7 buffer was added to each well and the plate was incubated for 5 minutes at room temperature. After incubation 200µl of 0.1M KH_2PO_4 pH6.9 was added to each well, followed by 29µl per well of 5mg/ml OPT in methanol. The plate was again incubated at room temperature for 30 minutes in the dark. After 30 minutes of incubation the plate was read using a Molecular Devices Spectra Max M5 fluorescent plate reader with parameters of excitation at 365nm and emission at 430nm, with a slit width of 5nm for each.

For total GSH readings, a black, flat bottomed 96 well plate was rinsed clean using distilled water prior to adding any reagents. Firstly, 19µl of 5% TCA in RQB was added per well, followed by 10µl of standard or sample. Following this 48µl 1M KPi pH 7 was added per well and the plate was allowed to incubate for 5 minutes at room temperature. In the fume cupboard, 7µl 10mM dithionite was added per well after 5 minutes and the plate was then allowed to incubate for 1 hour at room temperature. After this second incubation 200µl 0.1M KPi pH 6.9 was added per well, followed by 29µl 5mg/ml OPT in methanol per well. The plate was incubated for a further 30 minutes in the dark at room temperature. After incubation the plate was read as described previously.

Upon completion of a standard curve and establishing the GSH readings from each batch of worms it was decided that the pilot study should be run again with numerous batches of 5 and 10 worms in order to establish whether the batches would present with high variability.

4.2.2 Study 2: Pilot study with new Promega kit

A further pilot study was conducted using the "GSH-Glo Glutathione Assay" kit from Promega, U.K. The kit was stored at -20˚C away from light when not in use. Two hours prior to the beginning of the assay the kit was removed from the freezer and allowed to thaw at room temperature. Worms were removed from culture 12 days prior to testing and were physiologically synchronised as described in Chapter 2, Section 2.2.2. Worms were exposed to a number of concentrations of H_2O_2 (0 - 150µM; n = 3), with 5 worms per concentration. After a 96 hour exposure worms were checked for mortality. Any concentration that had a dead worm in it was disregarded for this study. Live worms were transferred from the vials into disposable petri dishes in order to be picked up using a metal pick. Worms were transferred via this metal pick (to minimise the amount of liquid taken up with them) into plastic vials containing 1ml of PBS + 2mM EDTA and weighed. After weighing, the worms and liquid were transferred into a round bottom tube using a plastic pastette. They were homogenised using a hand held homogeniser until no visible remains were left in the tube. The homogeniser blade was well washed in between each concentration by rinsing in tap water, alcohol and distilled water. Following homogenisation the solutions were transferred into 15ml falcon tubes using a pipette. The tubes were centrifuged for 10 minutes at 800rpm (136 g) and 4˚C. After this they were stored on ice. During centrifugation the reagents for the test were made up from the defrosted kit. The GSH-Glo Reagent 2x was made by mixing 33.3µl Luciferin NT, 33.3µl Glutathione-S-Transferase and 1.67ml GSH-Glo Reaction Buffer. The standards were made in sterile water using a GSH stock (5mM), ranging from 0 - 5µM GSH. When all reagents, worm solutions and standards had been made a white 96 well plate was loaded in duplicate with 10µl of standard and 50µl of sample was put into the appropriate wells. Following this 50µl of GSH Glo Reagent 2x was added to each well and the plate was incubated at room temperature for 30 minutes. After the first incubation, 100µl of reconstituted Luciferin Detection Reagent was added to each well and the plate was mixed briefly by hand. The plate was incubated for a second time at room temperature for 15 minutes in the dark. Following this the plate was read using a Molecular Devices Spectra Max M5 fluorescent plate reader with parameters of excitation at 365nm and emission at 430nm, with a slit width of 5nm for each.

In order to produce a standard curve for the data the average luminescence was calculated for each concentration. This average was then corrected by subtracting the control luminescence to remove the background level. Following this a scatter plot of the standards was produced in Excel which was then used to determine the graph equation and R^2 value for the data. The calculation for determining the amount of GSH in each sample was performed using the plot equation. The luminescence was divided by the y factor of the equation from the standard curve. After this the figure was converted from µM/L to nM/ml and divided by the wet weight of the tissue. This figure was expressed as nmoles/mg wet weight (WW) of tissue. Based on these pilot studies the full study protocol was developed.

4.2.3 Study 3: Full study with new Promega kit

Following the pilot studies it was decided that the kit was sufficient for use with a minimum of 5 worms per concentration. Worms were removed from culture 12 days prior to testing and were physiologically synchronised as described in Chapter 2, Section 2.2.2. Following this 12 day period worms were exposed in 20ml to 0, 2.5 and 10mg/L ZnO NPs and bulk particles (± 5mg/L HA) as described in chapter 3 (n = 3). At 0, 4, 8, 24, 48, 72 and 96 hours 5 worms were removed from each concentration. The preparation of worms, reagents and standards were prepared as described in Section 4.2.2. Following this the plate was loaded as described previously in Section 4.2.2.

4.3 Results

4.3.1 Experimental design

Table 4.2 below shows the experimental design for all experiments using the Promega kit in this chapter. The p value was accepted at 0.05, unless a Bonferroni Correction was applied (as detailed in each section).

Table 4.2: The experimental design of studies within this chapter.

Experiment	**Test**	**Factor**	**Factor**	**Interaction Factor**
H_2O_2	ANOVA	Concentration (2 + 1 Control)	-	-
NP vs. NPHA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	HA	Concentration HA
NP vs. NPHA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Time	Concentration Time
NP vs. NPHA	Scheirer-Ray-Hare	HA 2	Time	HA Time
Bulk vs. Bulk HA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	HA	Concentration HA
Bulk vs. Bulk HA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Time	Concentration Time
Bulk vs. Bulk HA	Scheirer-Ray-Hare	HA 2	Time	HA Time
NP vs. Bulk	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Particle	Concentration Particle
NP vs. Bulk	Scheirer-Ray-Hare	Time	Particle	Time Particle
NPHA vs. BulkHA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Particle	Concentration Particle
NPHA vs. BulkHA	Scheirer-Ray-Hare	Time	Particle	Time Particle

4.3.2 Studies 1: Pilot study using adapted protocol

A number of worm batches of various sizes were used when trying to ascertain what amount of tissue would be required in order to get a high enough reading of GSH to see a depletion, should there be any, when exposed to the ZnO NPs and bulk particles. A standard curve was produced using known quantities of GSH from 0 - 100µM (Figure 4.1). A high level of variability was seen across the reduced GSH levels in worm batches (Table 4.3) when using the adapted protocol. A high level of variability was also seen across the total GSH data set (Table 4.4).

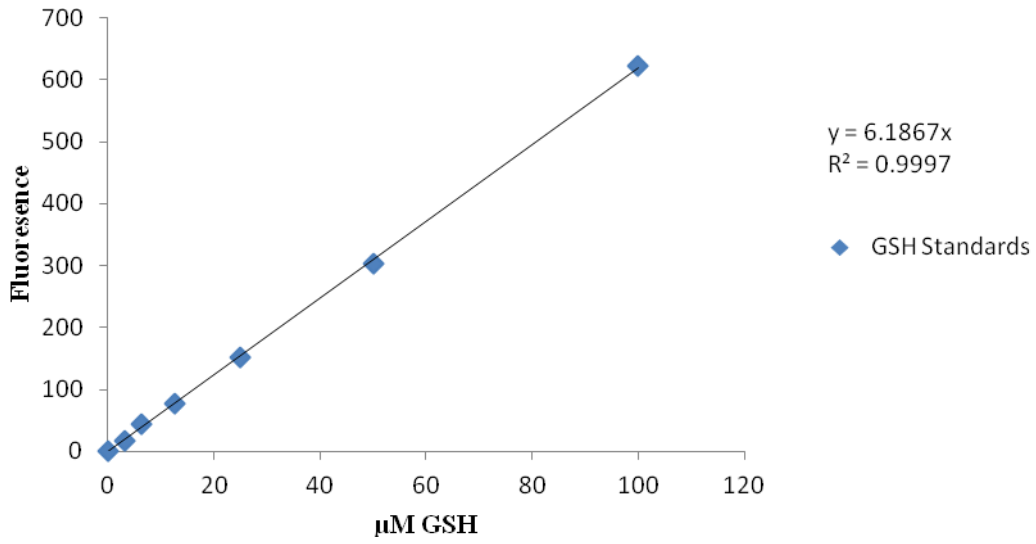

Figure 4.1: Standard curve of 0 - 100µM GSH.

Table 4.3: Average nmoles of reduced GSH/mg (WW) tissue for a number of worms (5 worms, n = 8, 10 worms, n = 8, 15 worms, n = 1, 20 worms, n = 2, 30 worms, n = 1).

	nmoles GSH/mg (WW) tissue				
	5 worms	**10 worms**	**15 worms**	**20 worms**	**30 worms**
Mean	0.00180	0.00048	0.00014	0.00453	0.00018
St. Deviation	0.00261	0.00051	-	0.00618	-

Table 4.4: Average nmoles of total GSH/mg (WW) tissue for a number of worms (5 worms, n = 8, 10 worms, n = 8, 15 worms, n = 1, 20 worms, n = 2, 30 worms, n = 1).

	nmoles GSH/mg (WW) tissue				
	5 worms	**10 worms**	**15 worms**	**20 worms**	**30 worms**
Mean	0.00073	0.00042	0.00015	0.00093	0.00021
St. Deviation	0.00141	0.00079	-	0.00101	-

The variability of the levels of GSH in worms using the adapted protocol was too high to continue with this protocol.

4.3.3 Study 2: Pilot study with new Promega kit

Based on the data gathered using the adapted protocol a kit was purchased from Promega, UK. H_2O_2 was used to induce a response in the levels of GSH within the worm tissue after 24 hours.

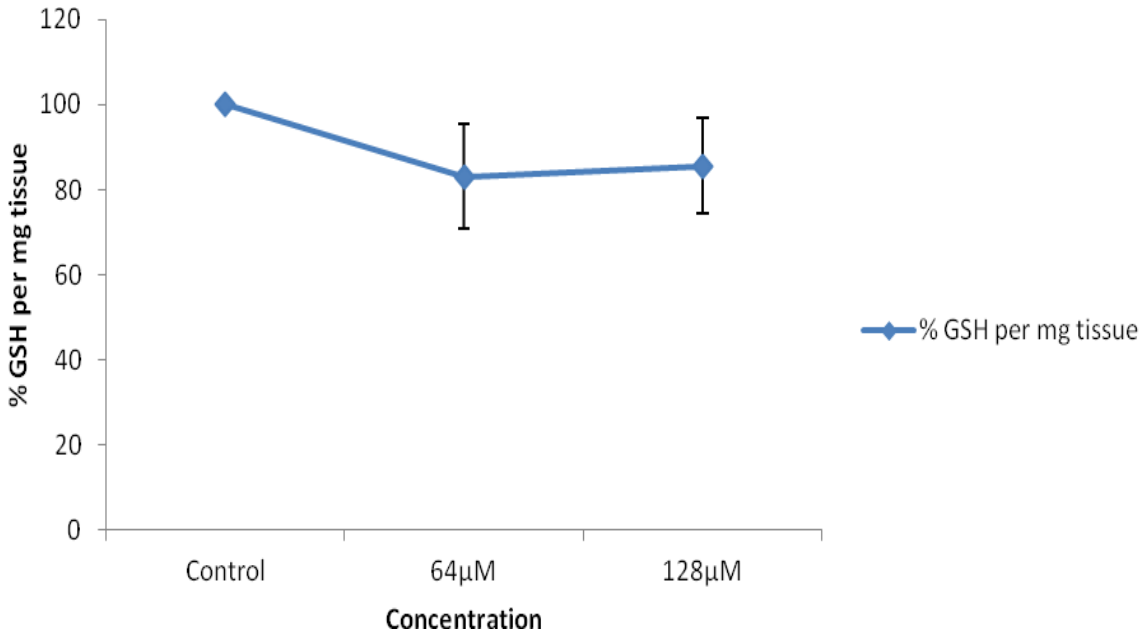

Figure 4.2.: Percent of GSH per mg of tissue in worms exposed to 0, 64µM and 128µM H_2O_2 for 24 hours (Data represents mean ± SE; n = 3).

Data were normalised to the control to show % GSH per mg of tissue with respect to the control worms. Data were tested for normality using a Kolmogorov-Smirnov test. The data were found to be normally distributed ($Z = 1.004$, $P > 0.05$) so a one way ANOVA was performed to test for any significant differences in percent GSH per mg tissue across the different H_20_2 concentrations (Table 4.5).

Table 4.5: Results from statistical analyses (ANOVA) conducted on percent GSH per mg tissue across the different H_2O_2 concentrations

Source	**d.f.**	**SS**	**MS**	**F**	**p value**
Percent GSH per mg tissue	2	501.090	250.545	0.908	0.452
Residual	6	1654.910	275.818		
Total	8	2155.999	269.500		

No significant depletion in GSH was observed in worms exposed to H_2O_2 for 24 hours (Figure 4.2).

4.3.4 Study 3: Full study with new Promega kit

Figure 4.3 shows the GSH levels in worms exposed to ZnO NPs over 96 hours, while Figure 4.4 shows the percentage GSH levels per milligram of tissue in worms exposed to ZnO NPs with 5mg/L HA, over the same time period. Data were normalised to the control to show % GSH per mg of tissue with respect to the control worms. Data were tested for normality using a Kolmogorov-Smirnov Test and were found to be not normally distributed ($Z = 3.223$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. Three Scheirer-Ray-Hare tests were performed as there is no non-parametric version of a 3 way ANOVA, to assess the influence of concentration, time and HA presence (Tables 4.6, 4.7 and 4.8). Bonferroni corrections were applied to account for the use of multiple tests.

Table 4.6: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO NP with and without addition of HA.

Factor	**SS**	**SS/MStotal**	**d.f.**	**F**	**p value**
Concentration	5483.556	5.589	2	6.170	0.061
HA	5662.259	5.772	1	2.987	0.016
Conc * HA	207.630	0.212	2	0.113	0.900

Table 4.7: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO NP at different time points.

Factor	SS	SS/MStotal	d.f.	F	p value
Concentration	5483.556	5.590	2	1.590	0.061
Time	7182.111	7.321	5	3.035	0.198
Conc * Time	11002.000	11.215	10	1.218	0.341

Table 4.8: Statistical analysis results obtained from Scheirer-Ray-Hare test assessing differences in time following exposures to ZnO NPs with and without HA.

Factor	SS	SS/Mstotal	d.f.	F	p value
HA	5662.259	5.771	1	6.427	0.016
Time	7182.296	7.321	5	1.630	0.198
HA * Time	7543.296	7.689	5	1.712	0.174

The Bonferroni correction indicated that the p value should be less than 0.0045. With this correction no factor was seen to be significant.

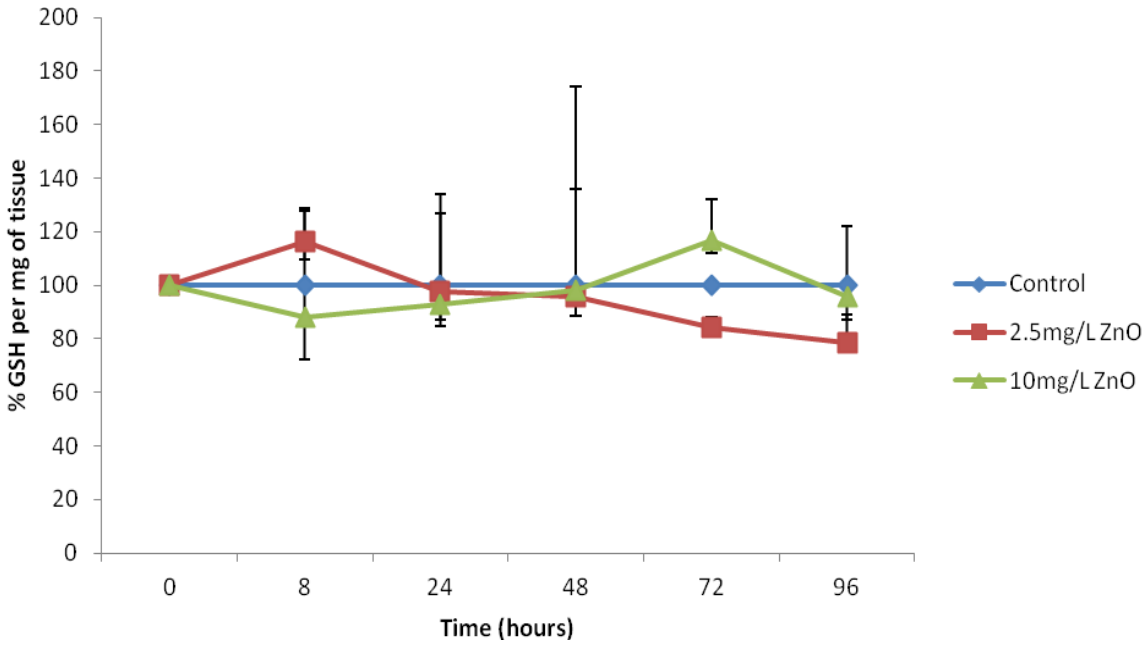

Figure 4.3: The percentage GSH per milligram of tissue in worms exposed to ZnO NPs over 96 hours (n = 3; data represents median with interquartile range).

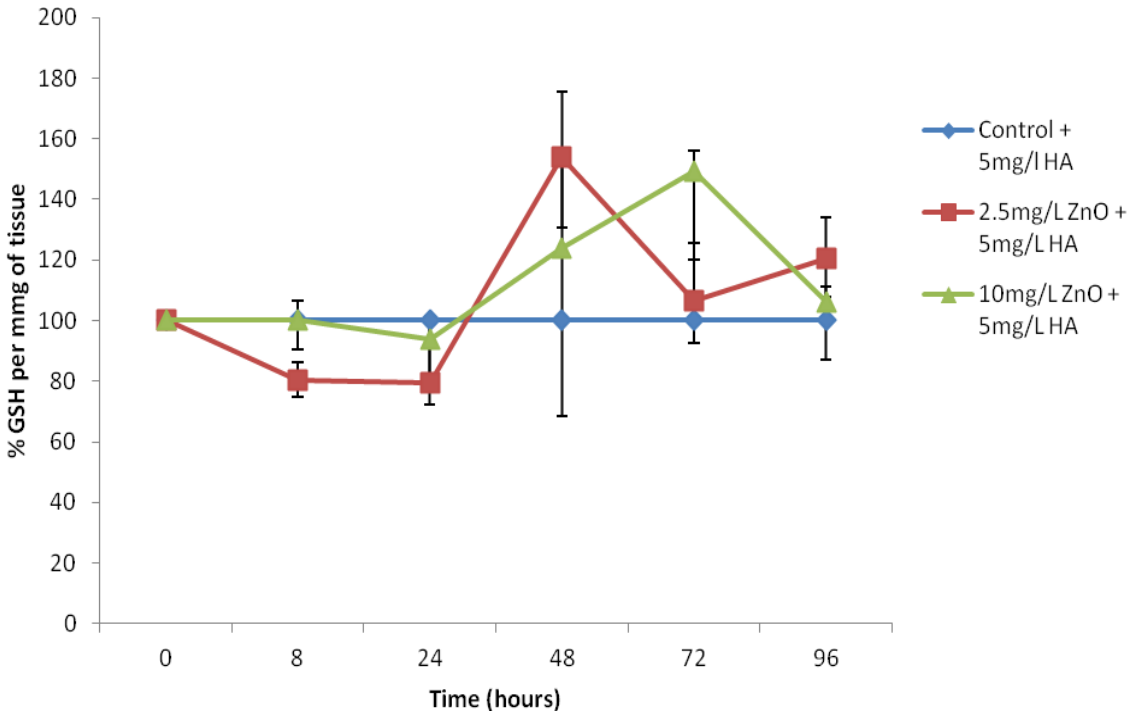

Figure 4.4: The percentage GSH per milligram of tissue in worms exposed to ZnO NPs with 5mg/L HA over 96 hours (n = 3; data represents median with interquartile range).

Figure 4.5 shows the GSH levels in worms exposed to ZnO bulk particles over 96 hours, while Figure 4.6 shows the percentage GSH levels per milligram of tissue in worms exposed to ZnO bulk particles with 5mg/L HA. Data was normalised to the control to show % GSH per mg of tissue with respect to the control worms. Data were tested for normality using a Kolmogorov-Smirnov Test and was found to be non-parametric ($Z = 2.640$, $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. Three Scheirer-Ray-Hare tests were performed (Tables 4.9, 4.10 and 4.11).

Table 4.9: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO bulk particles with and without addition of HA.

Factor	**SS**	**SS/MStotal**	**d.f.**	**F**	**p value**
Concentration	720.722	0.735	2	0.369	0.693
HA	56.333	0.057	1	0.058	0.811
Conc * HA	4447.722	4.534	2	2.274	0.104

Table 4.10: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO bulk particles at different time points.

Factor	SS	SS/Mstotal	d.f.	F	p value
Concentration	720.722	0.735	2	0.377	0.693
Time	9819.778	10.010	5	2.054	0.070
Conc * Time	8370.167	8.532	10	0.875	0.577

Table 4.11: Statistical analysis results obtained from Scheirer-Ray-Hare test assessing differences in time following exposures to ZnO bulk particles with and without HA.

Factor	SS	SS/Mstotal	d.f.	F	p value
Time	9819.778	10.010	5	2.223	0.075
HA	56.333	0.057	1	0.064	0.811
Time * HA	10287.556	10.487	5	2.239	0.063

From the 3 tests comparing concentration vs. HA, concentration vs. time and HA vs. time, it was found that there was no significant differences in GSH content of worms across ZnO NP and bulk particle exposure concentration, exposure length and the addition or omission of 5mg/L HA.

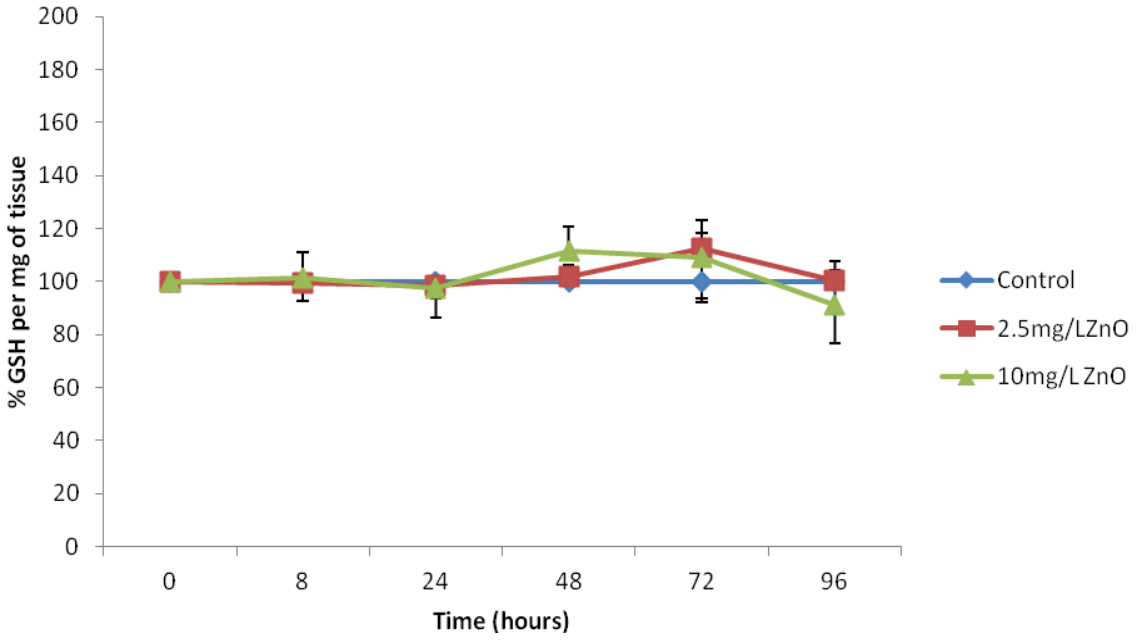

Figure 4.5: The percentage GSH per milligram of tissue in worms exposed to ZnO bulk particles over 96 hours (n = 3; data represents median with interquartile range).

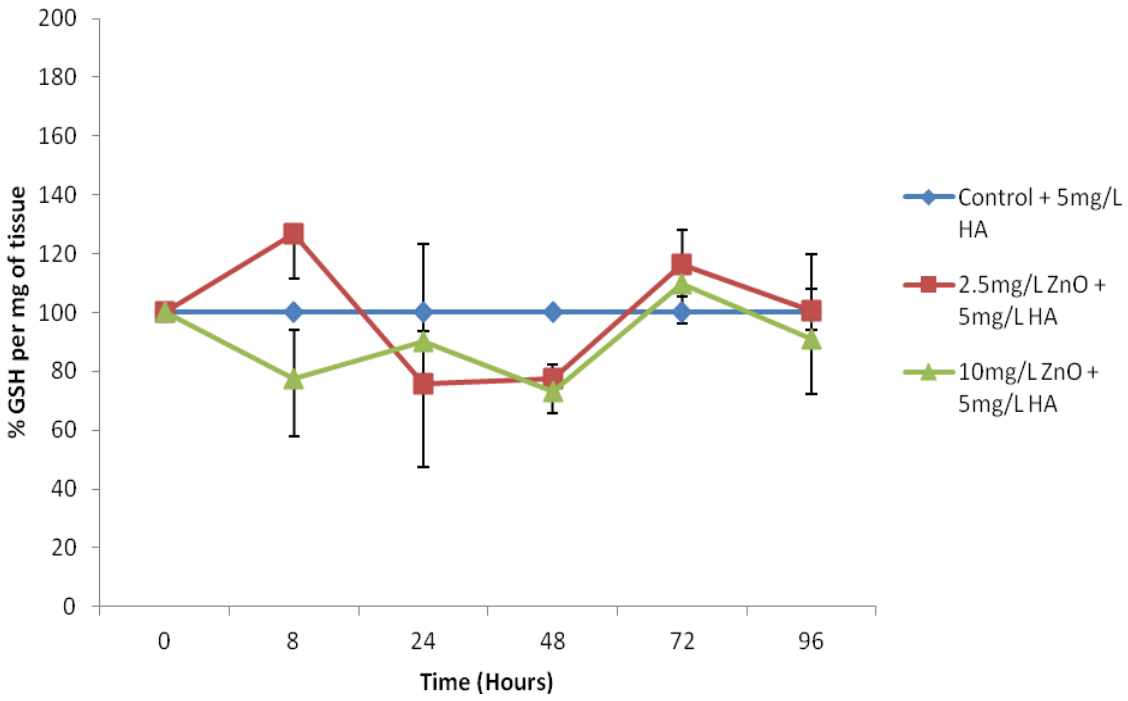

Figure 4.6: The percentage GSH per milligram of tissue in worms exposed to ZnO bulk particles with 5mg/L HA over 96 hours (n = 3; data represents median with interquartile range).

The NP and bulk particle data were investigated to assess if there was any significant difference in the content of GSH. Data were normalised to the control for each test. Data were tested for normality using a Kolmogorov-Smirnov Test and was found to be not normally distributed ($Z = 3.188$, $P < 0.001$). Two Scheirer-Ray-Hare tests were performed (Tables 4.12 and 4.13). Bonferroni corrections were applied to account for the use of multiple tests. This correction indicated that the p value should be less than 0.0045. With this correction no factor was seen to be significant.

Table 4.12: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO and particle type (NP and bulk).

Factor	**SS**	**SS/MStotal**	**d.f.**	**F**	**p value**
Concentration	189.061	0.192	2	0.100	0.908
Particle	3733.643	3.806	1	3.967	0.051
Conc*Particle	3626.335	3.697	2	1.927	0.158

Table 4.13: Statistical analysis results obtained from Scheirer-Ray-Hare test for time and particle type (NP and bulk).

Factor	**SS**	**SS/MStotal**	**d.f.**	**F**	**p value**
Time	6450.556	6.576	5	5.587	0.254
Particle	5152.926	5.253	1	1.399	0.022
Time*Particle	4816.630	4.910	5	1.044	0.427

The NP with HA and bulk particle with HA data was investigated to see if there was any significant difference in the depletion of GSH. Data has been normalised to the control for each test. Data was tested for normality using a Kolmogorov-Smirnov Test and was found to be non-parametric ($Z = 2.944$; $P < 0.001$).

Table 4.14: Statistical analysis results obtained from Scheirer-Ray-Hare test for concentration of ZnO with the addition of HA and particle type (NP and bulk).

Factor	**SS**	**SS/Mstotal**	**d.f.**	**F**	**p value**
Particle	204.402	0.208	1	0.226	0.648
Concentration	597.500	0.609	2	0.330	0.738
Conc*Particle	11958.760	12.190	2	6.600	0.002

Table 4.15: Statistical analysis results obtained from Scheirer-Ray-Hare test for time and particle type (NP and bulk) with the addition of HA.

Factor	**SS**	**SS/MStotal**	**d.f.**	**F**	**p value**
Particle	0.926	0.001	1	0.001	0.976
Time	8293.111	8.454	5	1.967	0.133
Time*Particle	15720.296	16.025	5	3.728	0.007

Two Scheirer-Ray-Hare tests were performed (Tables 4.14 and 4.15). Bonferroni corrections were applied to account for the use of multiple tests. This correction stated that the p value should be less than 0.0045. With this correction the interaction factor between the particle type and concentration was seen to be significantly different ($F = 6.6$; $d.f. = 2$; $P < 0.0045$) (Table 4.14), indicating that in this instance the effects of the two factors (particle type (with HA) and the effect of concentration) were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to other factor (Dytham, 2011). The two particle types responded

significantly differently in relation to the depletion of GSH in the cells depending on what concentration of particles the worms were exposed to.

4.4 Discussion:

4.4.1 Protocols

Two protocols were used in this study to ascertain which was the best method to use to investigate the effect of ZnO NPs and bulk particles on the GSH content of *L. variegatus* cells. The results suggest that the kit method was the best approach since the method was faster, the stability of the signal obtained was greater in the kit and the amount of tissue required for the kit method was far less than the adapted protocol. The advantages of using the kit protocol over the adapted protocol include the speed at which the kit worked (the adapted protocol took up to a full day to carry out whereas the kit took a few hours), the signal stability of the kit (the half-life of the signal in the kit was greater than 2 hours), the quantity of sample required (only 5 worms were required for the kit protocol whereas the GSH levels in the adapted protocol were highly variable across all worm sample sizes of 5 to 30 worms) and the lack of interference between fluorescent excitation and emission wavelengths of the samples and reagents (the kit was based on luminescence). The only disadvantage of using this kit was the high cost involved when compared to relatively low running cost of the adapted protocol.

4.4.2 Pilot Study

Hydrogen peroxide was used as a positive control in this study as it has been shown to cause oxidative stress as it is an ROS (Veal *et al.*, 2007), however it can also be an important factor in cell signalling. The response of multicellular organisms to H_2O_2 differs depending on the organism. The increased/decreased expression of antioxidants in response to H_2O_2 is not universal (An and Blackwell, 2003; Desaint *et al.*, 2004) and this suggests that in some cells it may be more important that antioxidant levels are set appropriately for H_2O_2 for cell signalling (Veal *et al.*, 2007) rather than responding to oxidative stress. H_2O_2 can therefore have both positive (signal) and negative (damage) effects and it is important to consider the organism or cell type under investigation. As the oxidative defences of *L. variegatus* are not well understood (Wiegand *et al.*, 2007)

and this kit has not been used with worm tissues before it was decided to continue with the study to examine whether ZnO NPs and bulk caused oxidative stress, even though there was no significant decrease in GSH levels in worms exposed to H_2O_2.

4.4.3 Oxidative stress study

The uptake, distribution, metabolism and elimination of toxic substances are key elements in determining the response of aquatic organisms to a substance (Verrengia Guerrero *et al*., 2002). In this study it was observed that ZnO NPs and bulk particles (± HA) were not seen to cause any significant oxidative stress in *L. variegatus* after 96 hours of exposure, using this methodological approach. A number of reasons may be put forward in order to potentially explain why an expected oxidative stress reaction was not observed. Lushchak (2011) suggested that pre-exposure to low intensity oxidative stress, no matter how that stress was induced, may enhance an organism's tolerance to a subsequent higher intensity oxidative stress. The pre-exposure in this case may have been that the worms may have incurred a low level stress within their culture tanks (e.g. reduction in oxygen supply/food or disturbance due to movement of the tank/light source) or during physiological synchronisation. It is also possible that the ZnO exposure concentrations were too low to produce enough ROS to induce lasting oxidative stress and the worms were able to compensate and readjust the amount of GSH after depletion. Thirdly, it has been stated that in studies using aquatic organisms changes in antioxidant mechanisms are transient and variable for different species and chemicals (Livingstone, 2001; Barata *et al*., 2005; Cochón *et al*., 2007). A significant increase/decrease and subsequent compensation in GSH levels may have occurred before the first investigative time point of 8 hours. Kumar *et al*. (2011) reported that oxidative stress occurred in *E. coli* after a 60 minute exposure to ZnO NPs and Huang *et al*. (2010) reported that oxidative stress occurred in human lung epithelial (BEAS-2B) cells after 6 hours. An early decrease in GSH levels could result in a short lived increase in GSH synthesis. This increase in GSH may have allowed the worm to adapt to the stress by detoxifying the toxicant and removing the ROS generated by it (Kristoff *et al*., 2008). Finally, it has been suggested that a number of biomarkers for oxidative stress should be used when investigating oxidative stress in aquatic organisms (Lushchak, 2011) and so just because there was no significant change in GSH levels it

cannot be fully concluded that no oxidative stress occurred. Knowledge of the regulation of antioxidants systems in aquatic organisms is limited in relation to environmental pollutants (Livingstone, 2001) and due to the conservative nature of the statistics used it would be beneficial to investigate the oxidative stress defences of *L. variegatus* further using GSH and other markers for oxidative stress, with a wider range of ZnO NP and bulk particle concentrations. The results of this study are not in line with ZnO toxicology studies (as described in Section 4.1.3) as oxidative stress upon exposure to ZnO has been measured in several human cell lines (e.g. De Beradis *et al.*, 2010; Huang *et al*., 2010; Fukui *et al*., 2012; Sharma *et al*., 2012) and environmental toxicity studies (e.g. Lin *et al*., 2009; Pujalte *et al*., 2011; Hao *et al*., 2012). However, in Hao *et al*. (2012) it was observed that after exposure to ZnO NPs the enzymatic and non-enzymatic antioxidant defences of carp (*Cyprinas carpio*) acted in different ways in different organs. The intestines were found to be relatively insensitive to the NPs when compared to the brain and liver. They suggested that this was proof that in order to fully evaluate oxidative stress biomarkers it is necessary to assess a number of markers in the organism. It can also be seen from Section 4.1.4 that the oxidative defence mechanisms in *L. variegatus* can differ depending on which marker is investigated.

4.5 Summary of this study

The GSH content of *L. variegatus* cells after exposure to ZnO NPs and bulk particles for 96 hours was investigated in this study. ZnO NPs and bulk particles (0 – 10mg/L with and without 5mg/L HA) were not found to have a significant effect on the level of GSH in the cells of the worms indicating that oxidative stress was not induced according to this marker. The aims of the study were met and it was shown that this protocol was successful in assessing the GSH content of *L. variegatus* cells after exposure to NPs and is a potential endpoint for assessing toxicity in *L. variegatus*, alongside other markers of oxidative stress, e.g. SOD, CAT, POD, etc. The Promega protocol was also considered successful as it reduced the amount of tissue and time required to conduct the study compared to adapted protocol initially used.

Chapter 5 Chronic Toxicity Testing: Investigation of the effects of ZnO nanoparticles and bulk particles on the behaviour of *Lumbriculus variegatus.*

5.1 Introduction:

Sediments are food sources and habitats for numerous organisms, and so, play a key role in aquatic ecosystems (Galluba *et al*., 2012). Sediment dwelling organisms themselves are food sources for many species and so a contamination of sediment can lead to effects in non-benthic organisms and even humans (Hansen *et al*., 2004; Erikson *et al*., 2010). The contamination of fresh water sediments is considered a major environmental concern for aquatic ecosystems (USEPA, 2002).

5.1.1 Sediment toxicology:

It has been recognised that sediments act as a sink for many contaminants and sediment feeders can accumulate high contaminant levels (Baun *et al*., 2008). Sediments can also act as sources of contaminants, as sediment bound contaminants can be released to the water column during periods of biological (Reible *et al*., 1996) and physical perturbation (Rubinos *et al*., 2010; Wu *et al*., 2011). Physical perturbation can include fast flowing waters, storm surges and anthropogenic influences (such as dredging), and bio-perturbation of sediment bound contaminants can occur, for example, via oligochaetes feeding on sediment particles and egesting/excreting them onto the sediment surface which can lead to a release of contaminants into overlying waters (Reible *et al*., 1996). In high densities benthic organisms can considerably rework and modify the structure of sediments (Krezoski and Robbins, 1985). Perturbation can lead to the contaminant being released continuously long after the initial entry of the contaminant into the environment (Galluba *et al*., 2012).

Sediment toxicity research began in earnest in the 1970s using lethality as an endpoint in amphipods and other crustaceans (Kalinowski and Zaleska-Radziwill, 2011). Standards began to appear during the 1990s which made use of further endpoints (e.g. reproduction, growth, biomass, bioaccumulation) and used a wider range of species (Kalinowski & Zaleska-Radziwill, 2011). An ideal sediment toxicity test

would: i) be ecologically relevant, ii) have the ability to assess chronic endpoints, iii) incorporate a wide range of routes of exposure (i.e. ingestion of particles, pore water, overlying water) and iv) be applicable to a wide range of sediment characteristics (Phipps *et al*., 1993). Sediment toxicity testing can be conducted using artificial or natural sediments, however natural sediments can exhibit numerous factors that can vary by location and often by season (Walsh *et al*., 1992), which can be problematic when used in toxicity studies. For example, natural sediment particles vary in surface area over orders of magnitude and in chemical composition, which can affect the nature and number of binding sites for metal and organic contaminants. The size of the natural sediment particles often define what species inhabit the sediment and the structure of the benthic community (Wenning *et al*., 2005). Finally, the sampling, transport and storage of natural sediments can change the composition of the sediment as well as the communities of organisms that may be living there (Reynolds *et al*., 1993; ASTM, 2002; Suedel and Rodgers, 1994). For this reason artificial sediments were used in this study as adapted from the OECD guidelines for the testing of chemicals 225 (2007). According to these guidelines an artificial sediment is preferred over a natural one as an artificial sediment minimises variability in the test conditions, the introduction of contaminants and the introduction of indigenous flora and fauna.

5.1.2 Use of Lumbriculus variegatus in sediment toxicity studies

According to Chapman *et al*. (1998) benthic organisms are the best indicators of sediment toxicity due to their contact with the sediment and overlying water. These organisms can be exposed to toxic substances in a number of ways including direct contact with the substance, contact via the pore water, ingesting contaminated food and contact via overlying water. The importance of the routes of exposure is dependent on the feeding and burrowing behaviour of the organism (Wenning *et al*., 2005). Burton (1992) listed a number of criteria for the selection of test organism for sediment toxicity testing including the behaviour of the organism in the sediment, ecological relevance, geographical distribution, sensitivity to contaminants, availability and tolerance to different sediment types. *L. variegatus* have been identified by the USEPA and OECD as a recommended freshwater organism for assessing the bioaccumulation of contaminants in sediments (USEPA, 2000; OECD, 2007). They often account for a large biomass in freshwater habitats, are a food source for secondary consumers

(Galluba *et al*., 2012), have a feeding strategy that brings them in direct contact with contaminants (Leppänen & Kukkonen, 1998) and can have a strong influence on the bioavailability of contaminants to other organisms (Egeler *et al*., 1997). These attributes make them a relevant species for the testing of contaminants. *L. variegatus* has been used to test the toxicity of a number of substances in artificial sediments (e.g. anti-parasitic drugs (Ding *et al*. 2001), metals (Hirsch, 1998; Gerhardt, 2007), organochlorines (Egler *et al*., 1997; Nikkilä *et* al., 2003; Mäenpää *et al*., 2008), polycyclic aromatic hydrocarbons (PAHs) (Leppänen & Kukkonen, 1998; Landrum *et al*., 2002) and steroids (Liebig *et al*., 2005), amongst others) and also in natural contaminated sediments (Sardo and Soares, 2010; Galluba *et al*., 2012). More recently *L. variegatus* has been used to investigate the toxicity of NPs. Petersen *et al.* (2008; 2010) investigated the bioaccumulation of carbon nanotubes (CNTs) in the worms and found that the CNTs were not absorbed into the tissues but were ingested by the worms. They indicated that more research was necessary, potentially using TEM imaging, to investigate this further. Parkarinen *et al*. (2011) invested the effects of nC_{60} fullerenes (carbon NPs) on *L. variegatus*. They found that feeding activity and depuration efficiency was decreased after exposure, however the effect was minimal. The authors suggested that although the NPs were not very toxic to *L. variegatus*, the worms ingested the NPs and egested them on to the sediment potentially increasing their bioavailability to other organisms. Hartmann *et al*. (2012) investigated the potential of TiO_2 NPs as carriers for uptake of cadmium in *L. variegatus*. They found that TiO_2 was found adhered onto the surface of the worm and also in the gut but exact locations were difficult to determine. They also found that TiO_2 NPs did not increase the toxicity of cadmium in the worms.

5.1.3 Metals in sediments

The bioavailability of metal and metal oxides and their potential toxicity are not only affected by their physicochemical properties but also by sediment associated factors including organic content, cation exchange capacity, pH, temperature, grain size (Förstner, 1990) and possibly acid sulphide content (Di Toro *et al*., 1990). Metals may partition in sediments in a number of ways including soluble free ions, organic and

inorganic complexes and precipitates of metal and inorganic complexes (Förstner, 1990; Di Toro *et al*., 1990).

In the literature many questions currently remain unanswered concerning the fate and behaviour of metal oxide NPs in sediments because in most instances the NPs are associated with the sediment matrix (Buffet *et al*., 2012). ZnO NPs are one of the most commonly used NPs in industry and inevitably some will be released to the environment. Studies have shown that ZnO NPs are prone to solubilise (NPs may have a greater rate of ion release due to their large surface area), increasing their bioavailability (Buffet *et al*., 2012). Bioavailability of contaminants is key in sediment toxicity testing. Only contaminants that are available to the organism can contribute to any toxic effect observed (Galluba *et al*., 2012). A study by Biddinger & Gloss (1984) indicated that organisms associated with sediments have higher zinc concentrations than organisms living in the water column. Zinc is more concentrated in sediments than in the water column and its content in sediments is closely correlated with depth, organic content and clay content of the sediment (ATSDR, 2005). Baun *et al*. (2008) stated that chronic, low exposure invertebrate NP toxicity tests are necessary as few studies have been published to date. The particles can come in contact with the worms via two pathways: i) NPs in the pore water can potentially enter the body via the integument or respiratory surfaces, or ii) sediment bound NPs can potentially enter the intestinal tract via sediment ingestion (Galluba *et al*., 2012). As the demand for ecological testing of contaminants has increased a further demand for the standardisation of protocols has emerged to ensure that studies are comparable (Van Geest *et al*., 2010).

5.1.4 Aims of the study

The main aim of this study was to investigate the effects of ZnO NPs and bulk particles on the mortality, reproduction and body reversal behaviour of *L. variegatus* in chronic (28 days) artificial sediment toxicity tests. The null hypothesis put forward for this study was that ZnO NPs and bulk particles would have no effect on the mortality, reproduction and body reversal behaviour of *L. variegatus* after a chronic exposure in artificial sediment toxicity tests. A further aim was to assess the impact of organic matter on the toxicity of ZnO NPs and bulk particles. The null hypothesis considered for this part of the study was that organic matter would have no impact on the toxicity

of ZnO NPs and bulk particles after chronic exposure to *L. variegatus*. The endpoints assessed in this study were the same as described in chapter 3. They included behavioural responses, which are a non-destructive (sub-lethal) method for assessing toxicity in *L. variegatus* (Warner, 1967; Lagadic *et al*., 1994). A final aim of the study was to investigate the use of paper substrate as a replacement for artificial sediment. The final null hypothesis considered was that a paper substrate would not be a sufficient replacement for artificial sediment.

5.2 Materials and Methods

This study was conducted in a number of steps in order to produce a protocol where worms survived until the end of the study. Firstly, studies were conducted for 28 days at Edinburgh Napier University, The Technical University of Denmark (DTU) and at Heriot Watt University (HWU) using a sediment protocol adapted from the OECD guidelines on the testing of chemicals 225 (2007). Following this, a study was conducted in HWU using the OECD 225 (2007) protocol. This protocol was followed exactly and was not adapted. Finally, a paper substrate study was conducted at HWU over a 28 day period.

5.2.1 Study Preparation

The medium used in these studies was as described in Chapter 3, Section 3.2.1. Worms were maintained as described in Chapter 3, Section 3.2.2. Equipment was maintained as described in Chapter 3, Section 3.2.3. Worms were physiologically synchronised prior to each experiment as described in Chapter 2, Section 2.2.2.

5.2.2 Study 1: Adapted sediment study

These studies were carried out at Edinburgh Napier University (n = 3), DTU (n = 3) and HWU (n = 3). The sediment used in this study was prepared according to an adaptation of the OECD guidelines on the testing of chemicals (OECD, 2007). The constituents of the sediment included cellulose (Sigma Alrich, UK), sand (Sigma Aldrich, UK), kaolinite (Sigma Alrich, UK) and ground nettle leaf (Napiers, UK) in the amounts shown in table 5.1.

Table 5.1: Constituents of adapted OECD sediment (225 Guidelines, 2007).

Constituent	**Amount**
Cellulose	4.5%
Sand	75.0%
Kaolinite	20.0%
Nettle leaf	0.5%

The constituents of the sediment were weighed out using a Sartorius LE244S (Napier/HW)/CP2245 (DTU) electric scales into a large plastic tub, according to Table 5.1. Prior to being weighed the nettle leaf was ground in a pestle and mortar and passed through 300µm mesh material. The sediment constituents were hand mixed gently for 15 minutes in the plastic tub. The nanoparticles and bulk ZnO were weighed out using the electric scales onto anti static trays in a laminar flow hood (DTU)/glove box (Napier; HW). Once weighed 0.005g ZnO particles were transferred to volumetric flasks containing 250ml EPA HW media and were sonicated in a sonication bath (Ultrawave Q series, 400W) for 30 minutes at 20˚C. The beakers were then placed on magnetic stirrers to ensure the NPs/bulk particle were dispersed as much as possible throughout the solution, before creating solutions of ZnO at 0, 500, 1000, 2000 and 4000mg/Kg (dry weight) sediment ± 5mg/L Suwannee River humic acid (both ZnO NPs and bulk particles).

Prepared sediment (200g) was weighed on the scale and 80ml of prepared nanoparticle suspension was added and stirred thoroughly through the sediment. The treated sediment was divided up into three 70g replicates and poured into three 250ml glass beakers. Each beaker was topped up with 150ml of EPA HW medium and the sediment was allowed to settle overnight. The following day the medium parameters were checked (Table 5.2).

Table 5.2: Aqueous medium parameters for testing during the assay.

Parameter	**Level**	**Checked**
Temperature	20°C ± 2°C	1 replicate once per week
Dissolved oxygen	> 30% saturation	1 replicate once per week
pH	6.0 – 9.0	1 replicate once per week

The dissolved oxygen was measured with a Hach LDO HQ10 O_2 dissolved oxygen (DO) meter. The temperature was also measured using the DO meter. The probe was inserted into the medium and gently stirred for 30 seconds until the percent oxygen and temperature stabilised. The pH was measured using a pHM210 standard pH meter (MeterLab Radiometer Analytical). The probe was gently stirred in the medium until the pH stabilised. After the parameters had been determined ten physiologically synchronised worms were added to each beaker. The beakers were placed at random in an incubator at 20 ± 2°C at Napier and HWU, or in a temperature controlled room at 20 ± 2°C at DTU, for 28 days and aeration was provided via air pumps which were used to bubble the beakers continuously. The parameters mentioned in section 5.2.2 were checked once per week. After 28 days the worms were removed from the beakers and mortality, reproduction and behavioural responses were assessed as described in chapter 3, section 3.2.5.

5.2.3 Study 2: OECD sediment study

This study was carried out at Heriot Watt University (n =3). NP and bulk suspensions were prepared as described in section 5.2.3. Suspensions of 0, 500, 1000, 2000 and 4000mg/Kg (dry weight) sediment ± 5mg/L Suwannee River humic acid were prepared. The sediment was prepared exactly as described in OECD guidelines on the testing of chemicals 225 (OECD, 2007) (Table 5.3).

Table 5.3: Constituents of OECD sediment (225 Guidelines, 2007).

Constituent	**% DW of sediment**
Sphagnum peat (air dried)	2 ± 0.5
Quartz sand	76
Kaolinite clay	22 ± 1
Nettle powder	0.4 - 0.5
Calcium Carbonate	0.05 - 1
Deionised water	30-50

Peat (Dow's Garden Supplies) was weighed and ground into a fine powder using a pestle and mortar. Visible plant remains were removed and the remaining peat was passed through a 500µM sieve giving a particle size of less than 0.5mm. A peat suspension was then made using a portion of the 30-50% deionised water (i.e. 690ml) that was to make up the final sediment and the pH was amended to 5.5 ± 0.5 using calcium carbonate. The sediment was gently mixed over 48 hours using a magnetic stirrer and the pH was measured again. The pH was amended to 6.0 ± 0.5 if necessary with $CaCO_3$. All other dry constituents were added along with the remaining deionised water. A homogenous sediment was obtained using a motorised mixer (Apollo APM 10 Mixer) for approximately 2.5 hours. After mixing, 300g of prepared sediment was treated with 120ml of prepared NP or bulk suspensions. Each particle concentration suspension was added to the sediment and mixed thoroughly. The spiked sediment was divided into 100g portions, added to 250ml beakers and 200ml of EPA HW medium (without particles) was added to each beaker. Beakers were then allowed to settle overnight. The following day the parameters were measured as described in section 5.2.2. After the parameters had been determined ten physiologically synchronised worms were added to each beaker. The beakers were placed at random in an incubator at 20 ± 2°C for 28 days and aeration was provided via air pumps which were used to bubble the beakers continuously. The parameters mentioned in section 5.2.2 were checked once per week. After 28 days the worms were removed from the beakers by gently heating the sediment in order to encourage the worms to come to the surface. Once at the surface the worms were gently removed using a plastic pastette. Mortality (determined as described in Chapter 3, Section 3.2.5), reproduction (determined by the number of worms remaining after testing - more than ten individuals was determined to constitute reproduction) and behavioural responses were assessed (as described in Chapter 3, Section 3.2.5).

5.2.4 Study 3: Paper sediment study

This study was carried out at Heriot Watt University (n = 3). NP and bulk solutions were prepared from 0 to 100mg/L ± 5mg/L humic acid as described in section 5.2.3. These concentrations were chosen based on the water only studies conducted as described in chapter 3, section 3.2.5. It was not possible to use the same exposure concentrations as the artificial sediments studies as these were too high. Positive controls were also set up for this experiment using 0.2 and 0.4µM $CuSO_4$ and 25 and 100mg/L $ZnSO_4$. O'Gara *et al.* (2004) showed that $CuSO_4$ was toxic to *L. variegatus* over various time points in a seven day period. It has also been shown that $ZnSO_4$ is toxic to a variety of oligochaetes (Callahan *et al*., 1994; Reinecke *et al*., 1996). The doses chosen for this study were based on the sublethal doses observed in O'Gara *et al*. (2004) and Callahan *et al*. (1994). (Positive controls with 5mg/L HA were not considered due to cost, time and space issues). Unbleached paper towels were used as substrate instead of an artificial sediment. Unbleached paper towels were washed twice in deionised water and autoclaved at 121˚C for 15 minutes before being cut into strips. Two paper towels (approximately 5g) were added into each beaker. NP or bulk suspensions (200ml) were added per beaker along with 1mg of pre-powdered nettle leaf. Parameters were checked as described in section 5.2.2. After the parameters had been determined ten physiologically synchronised worms were added to each beaker. The beakers were placed at random in an incubator at 20 ± 2°C for 28 days and aeration was provided via filtered air pumps which were used to bubble the beakers continuously. The parameters mentioned in section 5.2.2 were checked once per week. After 28 days the worms were removed from the beakers and mortality, reproduction and behavioural responses were assessed as described in chapter 3, section 3.2.5.

5.2.5 Statistical analyses

Statistical analyses were performed on the data using PASW 17.0 software. Data were checked for normality using a Kolmogorov-Smirnov test and any data transformations were performed if necessary and possible. Following this Scheirer-Ray-Hare or Kruskal Wallis tests were performed, given that data were found not to comply with parametric tests requirements (as described in Chapter 3, Section 3.2.9). Due to the use

of multiple tests a Bonferroni correction was applied. These statistics are highly conservative.

5.3 Results:

5.3.1 Study 1: Adapted sediment study

During testing using the adapted sediment at DTU, Edinburgh Napier University and HWU it was not possible to complete a 28 week study as sediments turned anoxic and worms did not survive. This study was repeated at Edinburgh Napier, DTU and HWU to assess if factors other than the sediment itself (e.g. water quality, contamination, incorrect mixing of sediment ingredients, differing batches of worms etc.) were affecting the protocol. It was not possible to complete the study in any of the three attempts. Figure 5.1 shows a beaker of sediment with worms at day 0 of testing. A part of one worm is visible burrowed into the sediment as expected, indicating that the sediment is initially suitable for the worms. Figures 5.2a and 5.2b show examples of sediments at day 28 of testing where worms are sitting on top of the sediment (Figure 5.2a) or are not visible (Figure 5.2b). Worms sitting on top of the sediment indicate perturbed conditions (OECD, 2007). The worms were not visible in Figure 5.2a as they were found to have died and decomposed after 28 days. The colour of the sediment can also be seen to have deteriorated after 28 days indicating that the sediments had become largely anoxic.

The O_2, pH and temperature were measured throughout all three studies as described in section 5.2.2 in each concentration of one replicate. All parameters were found to be within recommended OECD (2007) range (O_2 > 66% saturation, pH between 6 and 9, temperature 20 ± 1ºC) in each experiment throughout the 28 day exposure period.

Figure 5.1: Image of 500mg/Kg DW ZnO NP spiked adapted sediment with worms at day 0 of testing. A worm can be seen burrowing into the sediment.

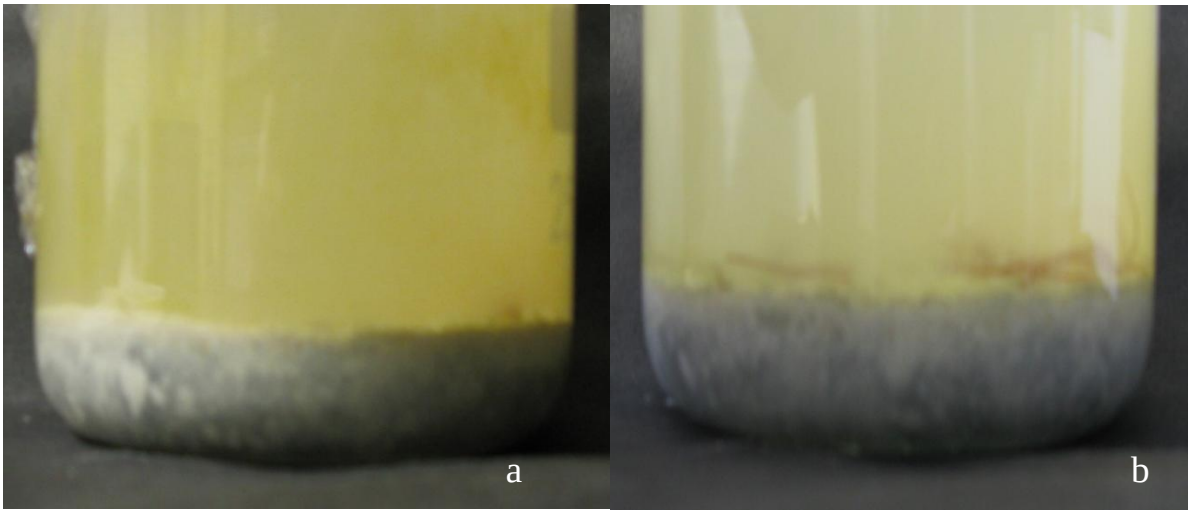

Figure 5.2: Images of NP spiked adapted sediments at day 28 of testing (a = 500mg/Kg DW ZnO NPs, b = 1000mg/Kg DW ZnO NPs). Worms can be seen sitting on top of the sediment in image 5.2b indicating that the sediment is unsuitable for their survival.

5.3.2 Study 2: OECD sediment study

This study was conducted at Heriot Watt University (n = 3), using concentrations of 0, 500, 1000, 2000 and 4000mg/Kg DW ZnO NPs and bulk particles with and without 5mg/L HA. Mortality in the control was found to be very high (EPA HW medium = 40%, EPA HW with HA = 57%) and of the control worms remaining the behavioural response was severely inhibited (EPA HW media = 15% behavioural response, EPA HW with HA = 14% behavioural response). Based on the controls this protocol was not continued.

5.3.3 Study 3: Paper sediment study

Table 5.4 below shows the experimental design for the paper sediment studies undertaken within this chapter. The p value was accepted at 0.05, unless a Bonferroni Correction was applied (as detailed in each section).

Table 5.4: The experimental design for the paper sediment studies.

Experiment	**Test**	**Factor**	**Factor**	**Interaction Factor**
$CuSO_4$	Kruskal Wallis	Concentration (2 + 1 Control)	-	-
$ZnSO_4$	Kruskal Wallis	Concentration (2 + 1 Control)	-	-
NP vs. NPHA	Scheirer-Ray-Hare	Concentration (4 + 1 Control)	HA	Concentration HA
Bulk vs. Bulk HA	Scheirer-Ray-Hare	Concentration (4 + 1 Control)	HA	Concentration HA
NP vs. Bulk	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Particle	Concentration Particle
NPHA vs. BulkHA	Scheirer-Ray-Hare	Concentration (2 + 1 Control)	Particle	Concentration Particle

This study was conducted at HWU (n = 3). Figure 5.3 below shows an example of the paper sediment.

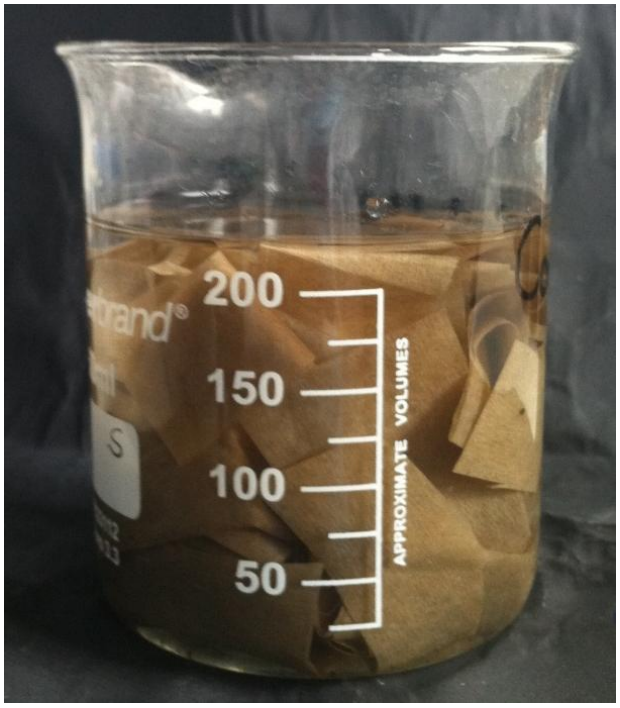

Figure 5.3: Image of the paper sediment.

Figure 5.4 shows the behavioural response data of worms exposed to NPs and NPs with HA at concentrations of 0, 12.5, 25, 50 and 100mg/L ZnO NPs and bulk particles with and without 5mg/L HA. Data were normalised to the test mean and outliers were removed using the modified Thompson Tau technique. Data were found to be not normally distributed using a Kolmogorov-Smirnov test ($Z = 2.101$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The effect of nanoparticle concentration and the addition of 5mg/L humic acid (HA) were investigated using a Scheirer-Ray-Hare test. The concentration of nanoparticles was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 72.992$, d.f. = 4, $P < 0.001$). The addition of 5mg/L HA and the interaction factor between concentration and the addition of HA were not found to be significant (table 5.5).

Table 5.5: Statistics obtained from the Scheirer-Ray-Hare Test comparing the effect of particle concentration and the addition of HA on the behavioural response of the worms.

Factor	F	SS	SS/Mstotal	d.f.	p value
Concentration	72.992	394222.827	39.402	4	<0.001
HA	0.187	1012.062	0.101	1	0.7500
HA*Concentration	1.508	8141.887	0.814	4	0.9400

Mann Whitney U tests were performed to investigate where the significances occurred. Bonferroni corrections were applied to account for the use of multiple tests. Control worms were found to have an expected behavioural response which was significantly different from all other concentrations ($P < 0.001$). With increasing concentration came a significantly negative behavioural response up to 50mg/L ($P < 0.001$). Worms exposed to 50mg/L and 100mg/L ZnO NPs were not seen to have a significantly different behavioural response from each other.

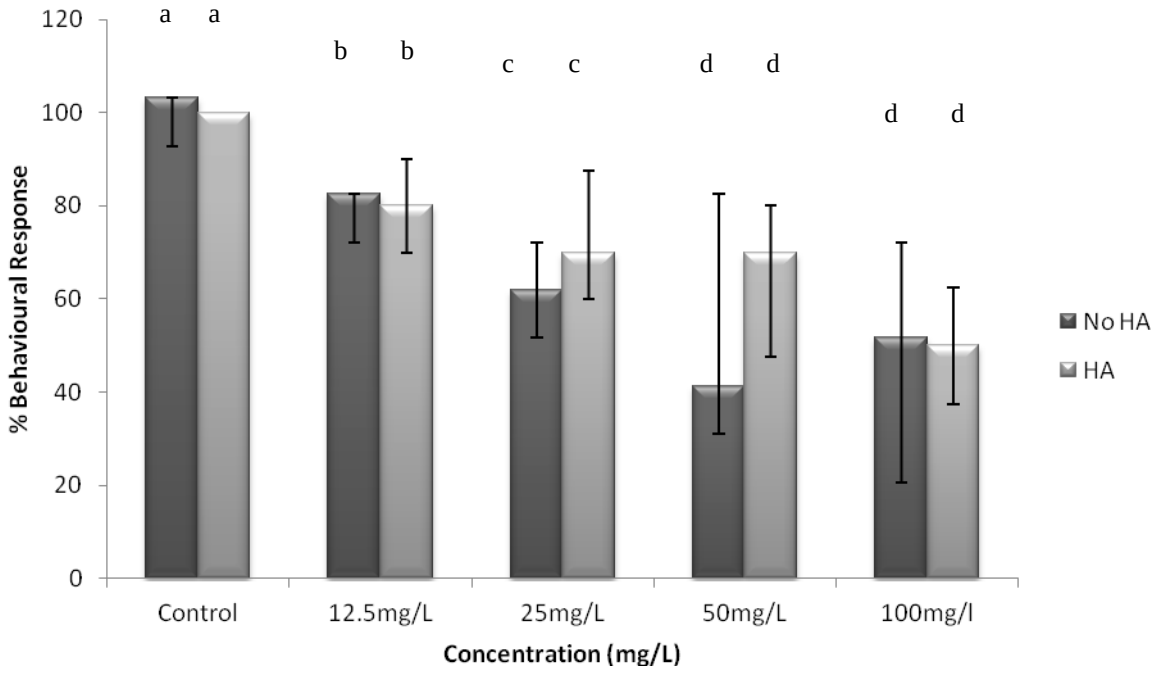

Figure 5.4: Percentage behavioural response of worms exposed to various concentrations of ZnO NPs and ZnO NPs with 5mg/L HA (data represent median with interquartile range; n = 3; a shared letter indicates no significant difference).

The behavioural response data of worms exposed to bulk particles with and without 5mg/L HA were statistically analysed in the same way as the behavioural response of worms exposed to ZnO NPs with and without 5mg/L HA. Data were found to be not normally distributed ($Z = 3.011$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The concentration of bulk particles was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 15.866$, d.f. = 4, $P < 0.01$). The addition of 5mg/L HA and the interaction factor between concentration and the addition of HA were not found to be significant (Table 5.6).

Table 5.6: Statistics obtained from the Scheirer-Ray-Hare Test comparing the effects of the concentration of bulk and the addition of HA on the behavioural response of the worms.

Factor	**F**	**SS**	**SS/Mstotal**	**d.f.**	**p value**
Concentration	15.866	71708.054	12.488	4	0.010
HA	1.428	6452.537	1.124	1	0.290
HA*Concentration	3.673	16601.537	2.891	4	0.580

Mann Whitney U tests were performed to investigate the significance observed in the result from the Scheirer-Ray-Hare test. Bonferroni corrections were applied to account for the use of multiple tests. Control worms had a significantly positive behavioural response when compared to all other concentrations ($P < 0.001$). With increasing concentration came a significantly worse behavioural response up to 100mg/L ($P < 0.001$). Worms exposed to 25mg/L and 50mg/L ZnO bulk particles were not seen to have a significantly different behavioural response from each other (Figure 5.5).

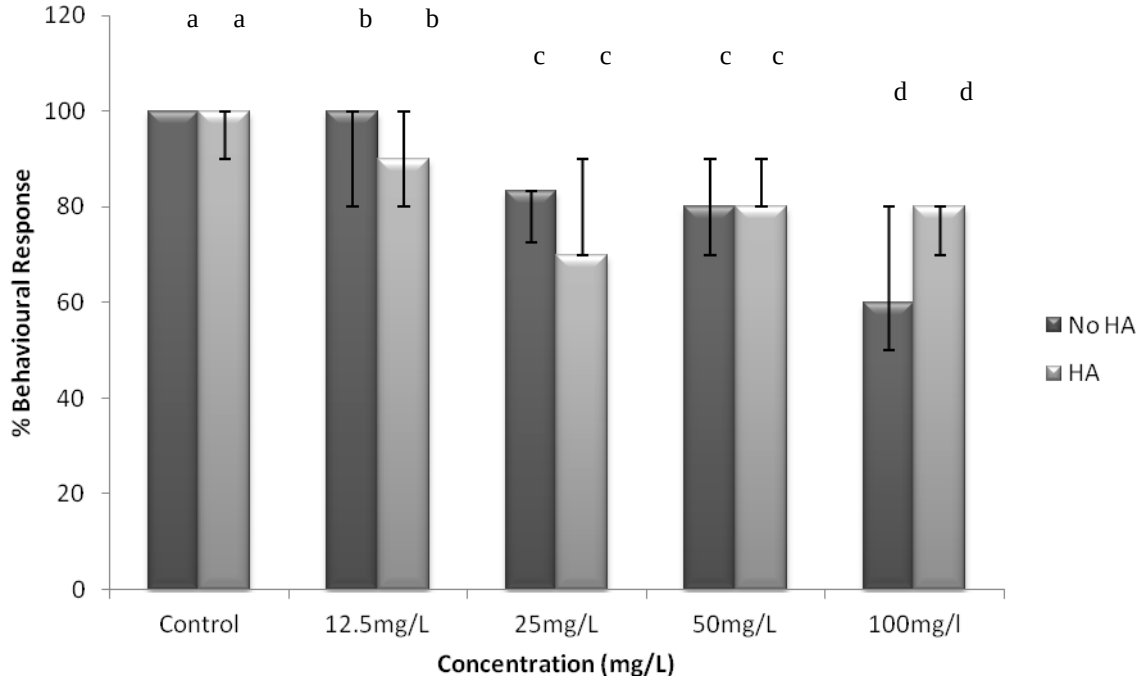

Figure 5.5: Percentage behavioural response of worms exposed to various concentrations of ZnO bulk particles and ZnO bulk particles with 5mg/L HA (data represent median with interquartile range; n = 3; comparison made on graph are between each and between each concentration and its corresponding HA concentration - a shared letter indicates no significant difference).

The NP and bulk data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms exposed to either particle type (Figure 5.6). Data were analysed in the same manner as previously described using normalisation to the control, outlier removal, normality testing, transformation and the Scheirer-Ray-Hare test. Data were found not to be normally distributed ($Z = 2.638$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The concentration of particles was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 62.215$, d.f. = 4, $P < 0.001$). The particle type was also found to be significant ($F = 42.298$, d.f. = 4, $P < 0.001$) (Table 5.7). Post hoc testing was performed using Mann Whitney U tests to investigate the significance observed in the Scheirer-Ray-Hare result. Bonferroni corrections were applied to account for the use of multiple tests. When comparing the particle type, the control worms were not found to have a significantly different behavioural response from each other. Worms exposed to

100mg/L ZnO NPs and bulk particles were also not found to be significantly different from each other, however, the 12.5mg/L, 25mg/L and 50mg/L NP exposures induced a significantly greater negative behavioural response when compared to 12.5mg/L, 25mg/L and 50mg/L bulk exposures ($P < 0.001$).

Table 5.7: Statistics obtained from the Scheirer-Ray-Hare Test comparing the effect of particle concentration (NP and bulk) and the addition of HA on the behavioural response of the worms.

Factor	**F**	**SS**	**SS/Mstotal**	**d.f.**	**p value**
Concentration	62.215	249798.379	29.924	4	<0.0001
Particle	42.298	169829.128	20.345	1	<0.0001
Concentration * Particle	9.088	36487.541	4.371	4	0.360

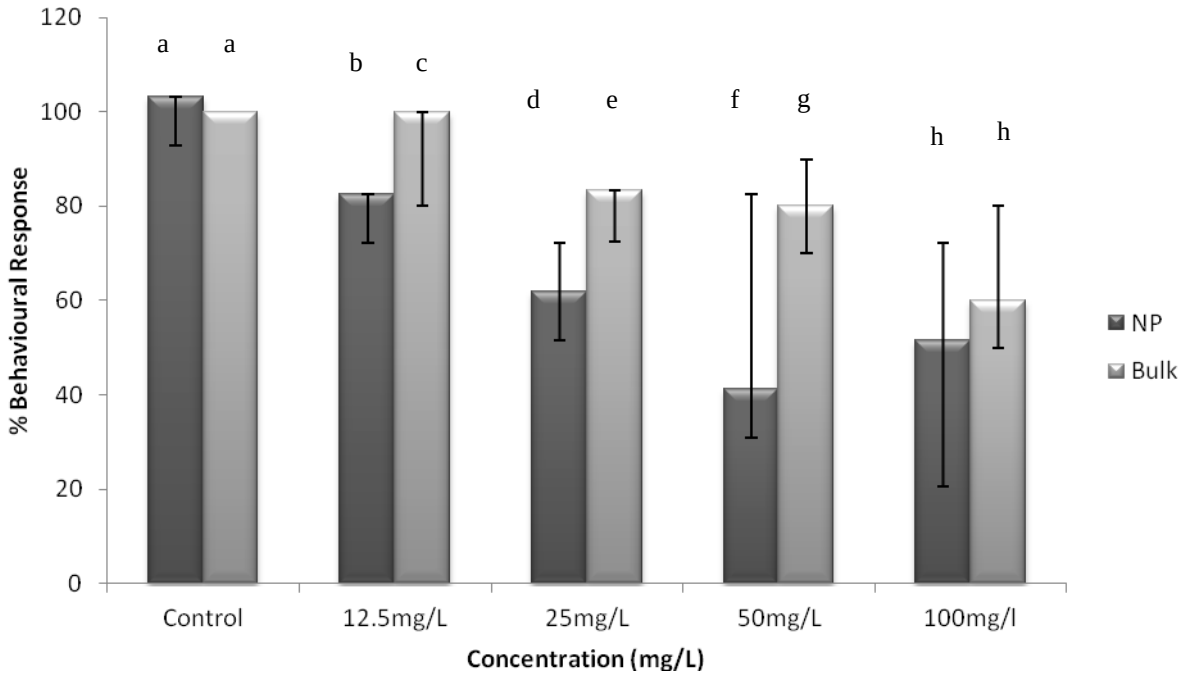

Figure 5.6: Percentage behavioural response of worms exposed to various concentrations of ZnO NPs and ZnO bulk particles (data represent median with interquartile range; n = 3; comparisons made for NP vs Bulk at each concentration - a shared letter indicates no significant difference).

Finally, the NP and bulk with 5mg/L HA data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms exposed to either particle type with 5mg/L HA (Figure 5.7). Data were analysed as described previously in the NP vs bulk comparison. Data were found not to be normally distributed ($Z = 2.421$; $P < 0.001$). Arcsine transformation was attempted however it was not possible to bring the data to normality. The concentration of particles was found to have a significant negative effect on the behaviour of the worms as the concentration increased ($F = 38.713$, d.f. = 4, $P < 0.001$). The particle type was also found to be significant ($F = 66.684$, d.f. = 4, $P < 0.001$) (Table 5.8). Since a significant effect was observed post hoc testing was performed using Mann Whitney U tests and Bonferroni corrections, as described previously.

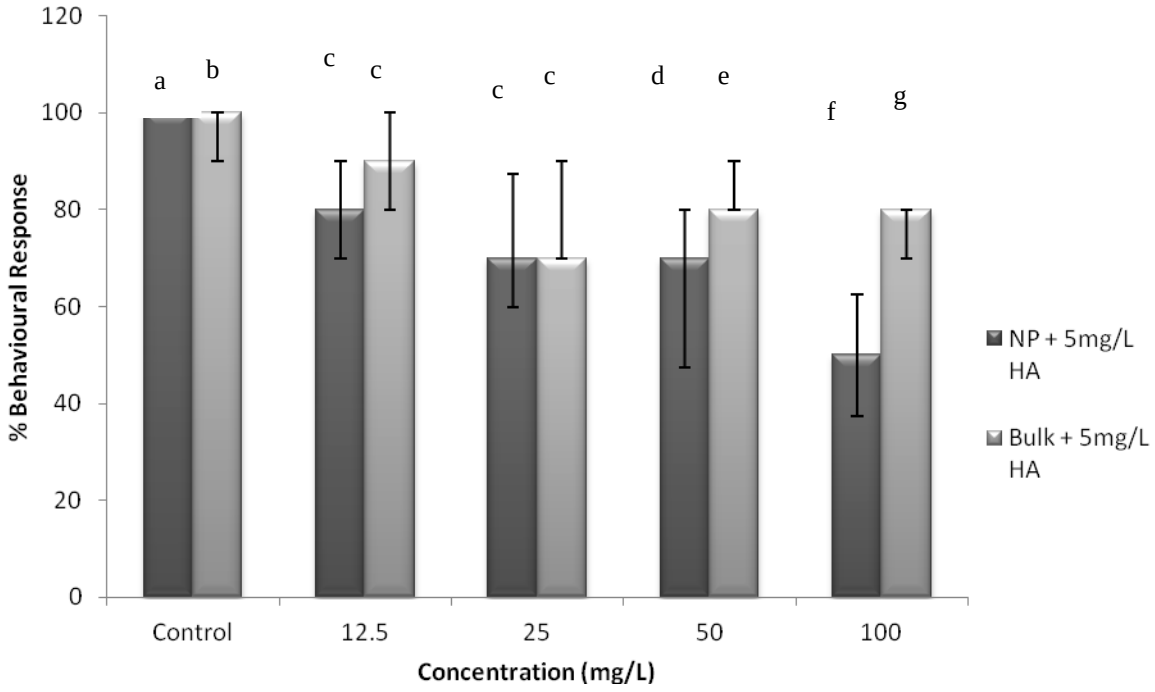

Figure 5.7: Percentage behavioural response of worms exposed to various concentrations of ZnO NPs with 5mg/L HA and ZnO bulk particles with 5mg/L HA (data represent median with interquartile range; n = 3; comparisons made for NP with HA vs Bulk with HA at each concentration - a shared letter indicates no significant difference).

Table 5.8: Statistics obtained from the Scheirer-Ray-Hare Test comparing the effect of particle concentration (NP with HA and bulk with HA) and particle type on the behavioural response of the worms.

Factor	F	SS	SS/Mstotal	d.f.	p value
Concentration	38.713	145660.249	20.430	4	0.0004
Particle	66.684	250901.340	35.191	1	<0.0001
Concentration * Particle	6.284	23643.725	3.316	4	0.506

The controls were found to be significantly different from each other, with the bulk HA control inducing a significantly greater negative behavioural response ($P < 0.005$) when compared to the EPA HW medium only control. Worms exposed to 12.5mg/L with HA and 25mg/L with HA were not found to be significantly different from each other, however worms exposed to 50mg/L with HA and 100mg/L with HA NP were found to induce a significant negative behavioural response ($P < 0.001$) when compared to the same concentrations of bulk.

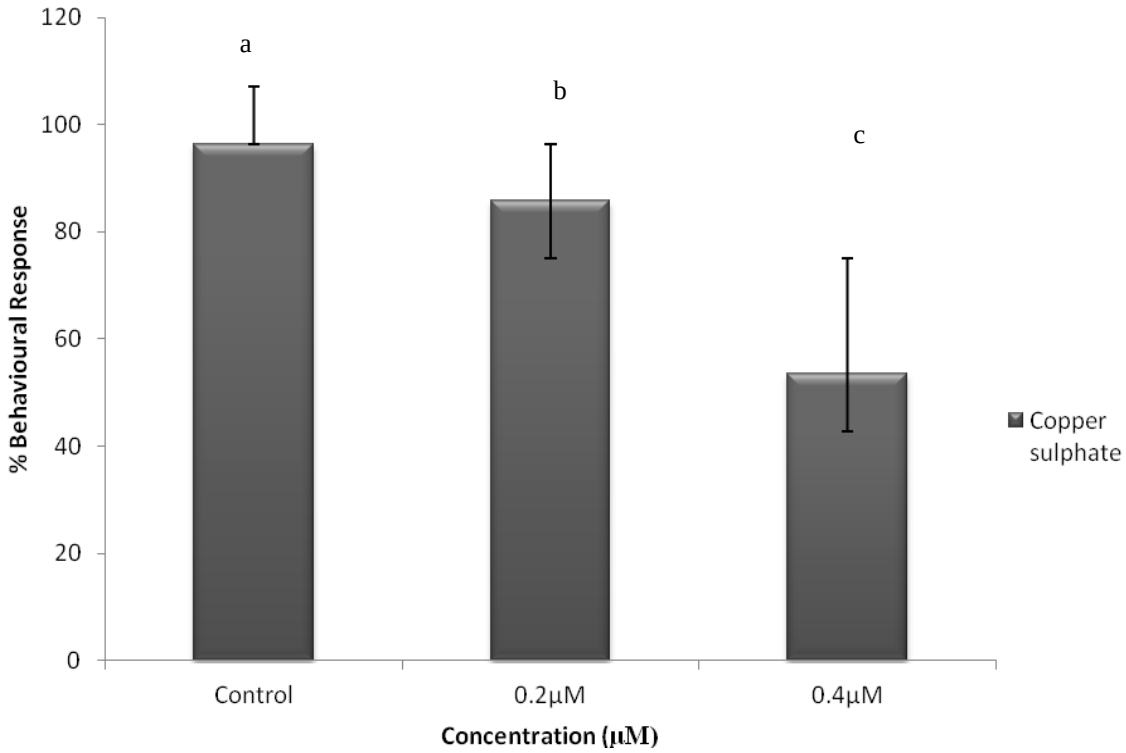

Figure 5.8: Percentage behavioural response of worms exposed to various concentrations of $CuSO_4$ for 28 days (data represent median with interquartile range; n = 3; a shared letter indicates no significant difference).

Figure 5.8 shows the behavioural response data of worms exposed to $CuSO_4$. Data were normalised to the test mean and outliers were removed using the modified Thompson Tau technique. Data were found to be not normally distributed using a Kolmogorov-Smirnov test ($Z = 1.871$; $P < 0.01$). Arcsine transformation was attempted however it was not possible to bring the data to normality. A Kruskal Wallis test was performed in order to assess if there was any significant difference in the behaviour of the worms across the concentrations. The test indicated that there was a significant difference between the concentrations ($P < 0.001$). Mann Whitney Tests were performed to see where these significances lay. A Bonferroni correction was applied to account for the use of multiple tests. The results indicated a dose dependent negative response with worms exposed to 0.4µM $CuSO_4$ having a significantly more negative response than the control and 0.2µM worms ($P < 0.001$).

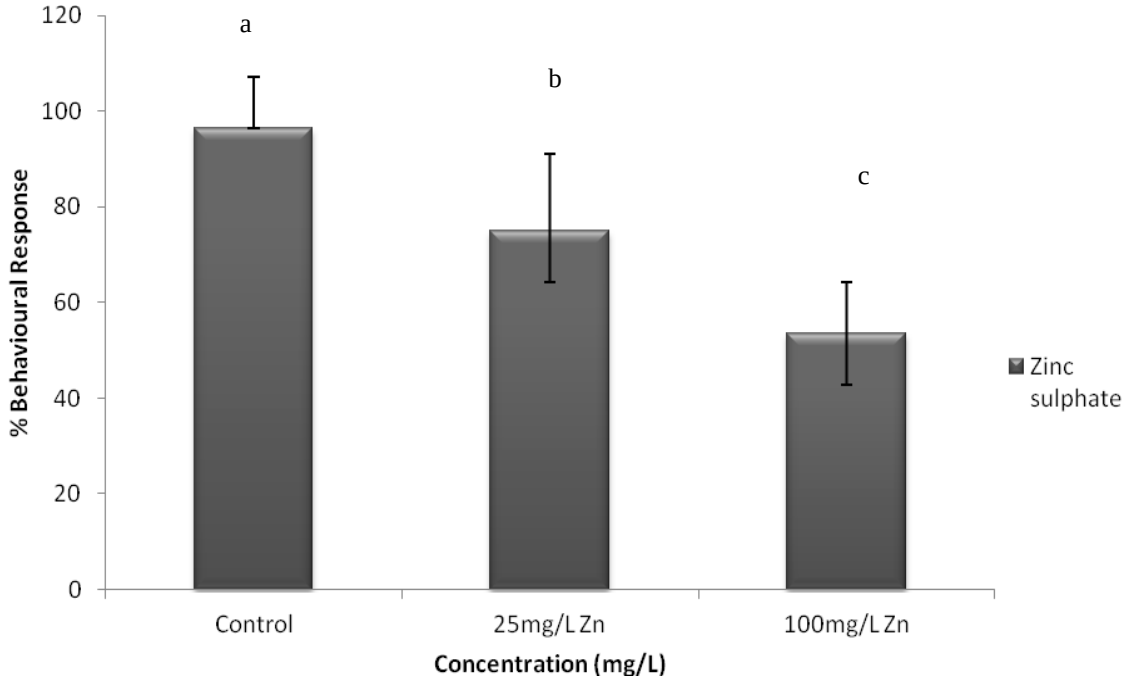

Figure 5.9: Percentage behavioural response of worms exposed to various concentrations of $ZnSO_4$ after a 28 day exposure (data represent median with interquartile range; n = 3).

Figure 5.9 shows the behavioural response data of worms exposed to $ZnSO_4$. Data were normalised and outliers were removed as described in previous sections. Data were found to be non-parametric ($Z = 1.658$; $P < 0.01$). Arcsine transformation was attempted however it was not possible to bring the data to normality. A Kruskal Wallis test was performed in order to assess if there was any significant difference in the behaviour of the worms across the concentrations. The test indicated that there was a significant difference between the concentrations ($P < 0.001$). Mann Whitney Tests were performed to see where these significances lay. A Bonferroni correction was applied to account for the use of multiple tests. The results indicated a dose dependent negative response with worms exposed to 100mg/ L $ZnSO_4$ having a significantly more negative response than worms exposed to 25mg/L and the control ($P < 0.001$).

5.4 Discussion

5.4.1 Artificial sediment studies

Obtaining the optimum conditions for sediment toxicity testing is a difficult and complex process (Arrate *et al*., 2004). Despite the amount of work conducted on the toxicity of NPs in both freshwater and sea water there has been relatively little work done on the toxicity of NPs in sediments (Pang *et al*., 2012). An important challenge that has potentially limited the investigations of NPs in sediments is the lack of a method to quantify, characterise and describe their fate in environmentally relevant media (Petersen *et al*., 2008) and it has been stated that there is a great need for characterisation techniques that are capable of quantifying and characterising NPs in environmental media (Baun *et al*., 2008). The use of an adapted sediment was not successful during these studies. This sediment was devised based on the OECD recommended artificial sediment (2007). The adaptations were included to avoid any potential contamination or variation that may have been introduced by the addition of peat into the sediment. Metals tend to naturally concentrate in sediments and peat (Owen *et al.,* 1992) and should there have been a background level of metal in the peat it may affect the results obtained in a toxicity test. Variability may be introduced depending on the original source of the peat. Worms did not survive the full 28 days of testing and those that did in the controls did not behave as expected, indicating stressful

conditions. The lack of peat may have resulted in a lack of essential factors for survival of the worms in the artificial sediment, e.g. factors such as lack of sufficient amounts of organic matter, sediment composition and grain size. Gerhardt (2007) stated that *L. variegatus* grew better in fine and medium sediment compared to coarse and whole sediments. There may also not have been sufficient food in the sediment (it was difficult to ensure that the nettle leaf remained in the sediment and not in the overlying water). The OECD sediment study was also not successful. A potential explanation of why this artificial sediment failed may have been that ammonia was produced within the sediment. The natural decomposition of the organic matter in the sediment can lead to high levels of ammonia in the pore water and overlying water during the first few days after preparation which can be a confounding factor in artificial sediment toxicity testing and may cause test failures due to poor survival rates in the controls (Arrate *et al*., 2004). Schubauer – Berigan *et al*. (1995) showed that ammonia at 24mg/L caused 60% mortality in *L. variegatus* and at 34.6mg/L no survival was observed after a 10 day exposure, at pH 8, while a study by Whiteman *et al*. (1996) reported that *L. variegatus* avoided the sediment because the pore water had higher levels of ammonia that the overlying waters. Arrate *et al*. (2004) suggested that artificial sediments should be allowed to stand for at least two weeks (with a pH maintained at 8 to avoid toxicity caused by unionised ammonia) after preparation to allow for ammonia reduction in the water column and pore water. The overlying water should then be removed and the sediments should be treated with the contaminant of interest and used for testing. The OECD (2007) protocol does suggest that a conditioning period of seven days, at pH below 7, however it does not state that it is essential. The present study had one day of conditioning and the sediment to water ratio was kept at 1:4 (respectively) which was stated in the protocol to avoid the build up of ammonia. In order to improve on the present study, should it be conducted again, a longer conditioning period between seven and fourteen days should be considered, along with testing for ammonia. It would also be recommended to use the OECD (2007) medium as opposed to EPA HW medium. Testing recently conducted using OECD sediment and OECD medium, as well as a slight reduced organic (peat) content (by Simon Little, a PhD student within the nanosafety research group) has proven to be successful, with worms surviving and reproducing well in the controls after 28 days.

5.4.2 Paper sediment study

This study was carried out in order to investigate whether a paper substrate could be used as a replacement for artificial or natural sediments when testing contaminants in *L. variegatus*. These conditions are normally used in worm cultures and therefore it is clear that they are suitable for worm survival and reproduction. The positive controls used in this study indicated that the protocol was valid as a dose dependent negative effect was observed in the behaviour of worms exposed to $CuSO_4$ and $ZnSO_4$ after 28 days, with very little mortality. This relates to the acute positive control studies (Chapter 3, Section 3.3.3) where a dose dependent negative effect was also observed in the behaviour of worms exposed to $CuSO_4$ and $ZnSO_4$ after 96 hours. It was found that ZnO NPs had a dose dependent negative effect on behaviour up to 50mg/L, ZnO bulk particles had a dose dependent negative effect on behaviour up to 100mg/L and the addition of 5mg/L humic acid had no effect on the toxicity of either particle type. When comparing NP toxicity to bulk toxicity, it was found that the NPs induced a greater negative effect on behaviour than the bulk particles. As discussed in the acute toxicity studies (Chapter 3, Section 3.4.3), the larger surface area and greater solubility of the ZnO NPs, compared to the ZnO bulk particles, may have a played a role in the differences in observed toxicity. However, unlike in the acute studies, the bulk particles were seen to induce a negative effect on behaviour and the HA did not affect the toxicity of either ZnO particle type. Within the acute toxicity studies it was found that HA mitigated the toxic effects of ZnO NPs and bulk particles over 96 hours and it was expected that this occurred due to the increased dispersion and stability of the particles in the water column. A number of studies have stated that natural organic matter (NOM) can increase the dispersion of NPs in aqueous media (silver NPs, Gao *et al*., 2012; carbon nanotubes, Chappell *et al*., 2009, carbon nanotubes, Lin and Xing, 2009), however Gao *et al*. (2012) also stated that at higher levels of NOM (i.e. >10mg/L) agglomeration and aggregation of silver NPs can increase. Due to the addition of nettle leaf in this study it is possible that the NOM content of this study was increased anyway. If agglomeration and sedimentation of the particles were increased, the worms (which stayed at the bottom of the test vessel within the paper "sediment") would have come in contact with the agglomerates which would not have been the case in the acute studies. The time factor may have also played a role in the different toxicity observed in the acute studies (96 hours) and the chronic studies (28 days). Finally, the

introduction of food to this test may also have had an impact on the toxicity of the particles (± HA). The particles may have adsorbed onto the nettle leaf leading to agglomeration and sedimentation of particles.

These studies were conducted in order to ascertain whether this method could be put forward as an alternative to the sediment studies. While these studies provide preliminary data on the chronic toxicity of ZnO NPs and bulk particles they do not necessarily accurately represent an ecologically relevant situation. However, this method may be a quicker (in terms of preparation) and cheaper alternative to the sediment toxicity test, where the test media can be sampled over time to examine the changes in NP physicochemical characteristics during a chronic study. This is not something that is done in sediment NP toxicity tests as there is currently no technique to characterise the particles in sediments.

5.5 Summary of this study

This study investigated the effects of ZnO NPs and bulk particles (with and without 5mg/L HA) on the mortality, reproduction and body reversal behaviour of *L. variegatus* in chronic artificial sediment toxicity tests and artificial paper sediment toxicity tests. Artificial sediment studies were not brought to completion as worms did not survive the 28 day exposure however in the paper sediment study was successful and both ZnO NPs and bulk particles (0 – 100mg/L with and without 5mg/L HA) were found to have a significant negative effect on the behaviour of the worms. The addition of HA had no effect on the toxicity of the particles which is a contrast from the acute behavioural study. NPs were found to have a greater negative effect when compared to bulk particle toxicity. The larger surface area and greater solubility of the ZnO NPs, compared to the ZnO bulk particles, may have a played a role in the differences in observed toxicity. The paper sediment protocol was developed as a replacement assay for the artificial sediment test. This protocol was successful in provided preliminary data on the chronic effects of ZnO NPs and bulk particles and is a useful assay for determining chronic toxic effects in *L. variegatus*.

Chapter 6 Depuration Study: Investigation of the uptake and depuration of ZnO nanoparticles and bulk particles in *Lumbriculus variegatus*.

6.1 Introduction

Many NPs currently used in industry contain metals and metal oxides and so the toxicity and bioaccumulation of metals in aquatic species may become an important environmental issue. Although the bioavailability of metals from NPs may be lower than those present in the environment in soluble form, the physicochemical properties of the NPs may be significant in terms of bioavailability and toxicity of metals (Bystrzejewska-Piotrowska *et al*., 2009).

6.1.1 Bioaccumulation of contaminants in aquatic species

Many environmental contaminants have the potential to accumulate through the aquatic food chain which can expose higher trophic levels to the contaminant, including humans (Dawson *et al*., 2003; Krysanov *et al*., 2010). Contaminants may be taken up by an organism in a number of ways (Zhu *et al*., 2006; Templeton *et al*., 2006; Elias *et al*., 2007). Firstly, the material may adsorb onto the exterior of the organism (Handy & Eddy, 2004). Secondly, the material may be taken up across the cell membrane, however the mechanisms for this in aquatic organisms are not yet fully understood (Handy *et al*., 2008). These mechanisms may include the dissolution of the substance and aqueous uptake of ions, diffusion of hydrophobic substances across the cell membrane or by endocytosis of the substances into the cell (Handy *et al*., 2008). Variability in the bioaccumulation of contaminants can occur in various species and may be due to a number of factors such as differences in route of exposure (Leppänen & Kukkonen, 2004), uptake rate (Millward *et al*., 2001), digestion time (Wang and Chow, 2002), desorption efficiency (Smoot *et al*., 2003), absorption efficiency (Wang and Fisher, 1999), metabolism (Gaskell *et al*., 2007) and excretion (Verrengia Guerrero *et al*., 2002). In addition some benthic organisms selectively feed on certain particle sizes and also some actively avoid contaminated locations (Millward *et al*., 2001; Kukkonen & Landrum, 1994).

6.1.2 The use of Lumbriculus variegatus in depuration studies

Oligochaetes represent almost 60% of the test species in aquatic bioaccumulation studies, with *L. variegatus* being the most popular choice (Van Geest *et al*., 2010). The OECD 225 guideline (2007) and USEPA (2002) have identified *L. variegatus* as a recommended test organism to assess the bioaccumulation of contaminants. As mentioned in chapter 5, section 5.1.2, *L. variegatus* has been used to test the bioaccumulation and toxicity of a number of substances, e.g. cadmium and nickel (Ankley *et* al., 1994), silver (Hirsch, 1998), lead (Miño *et al*., 2006), organochlorines (Egeler *et al*., 1997; Nikkilä *et* al., 2003; Mäenpää *et al*., 2008), PAHs (Leppänen & Kukkonen, 1998) and steroids (Liebig *et al*., 2005). *L. variegatus* has also been used to investigate the bioaccumulation and toxicity of some nanomaterials, such as carbon nanotubes (Petersen *et al.* 2008; 2010), nC_{60} fullerenes (Parkarinen *et al*., 2011) and TiO_2 NPs, as potential carriers of cadmium (Hartmann *et al*., 2012). All of these studies found that the NPs were taken up by the worm via ingestion, with some depuration of the NPs taking place over time.

6.1.3 Zinc oxide nanoparticle bioaccumulation studies

A number of authors have shown that ZnO NPs are prone to solubilise, increasing their bioavailability (Buffet *et al*., 2012). A critical factor which may affect the bioavailability of metal oxide NPs is the release of metal ions (Wong *et al*., 2010) and a further factor is the amount and type of organic matter that the NP encounters in the environment (Hyung *et al*., 2007). Recently, a number of papers have reported the bioaccumulation of ZnO NPs in a variety of species. ZnO NPs have been shown to be taken up by a number of plant species, such as, *Glycine max* (Soy bean; Lopez-Moreno *et al*., 2010; Priester *et al*., 2012), *Tritium aestirum* (Wheat; Du *et al*., 2011), *Juliflora velutina* (Desert plant; Hernandez-Viezcas *et al*., 2011) and *Fagopyrum esculentum* (Buckwheat; Lee *et al*., 2012). ZnO NPs have also been shown to bioaccumulate in animal species, such as *Danio rerio* (zebra fish; Yu *et al*., 2011) and *Lymnea stagnalis* (freshwater snail; Croteau *et al*., 2011).

From the above studies it has been shown that NPs can enter the body of an aquatic organism, however their migration through the food chain has not yet been

evaluated (Krysanov *et al*., 2010). This study does not investigate food chain effects of NP uptake/depuration however it has been suggested that if contaminants are accumulated within the food chain of aquatic species it can cause physiological impairment at higher trophic levels, even in human consumers (Freitas *et al*., 2012) and so the understanding of the uptake, accumulation and depuration of NPs is of great importance.

6.1.4 Aims

The main aim of this study was to investigate the uptake, accumulation and depuration of ZnO NPs and bulk particles in *L. variegatus* over a 48 hour period of uptake and a 48 hour period of depuration. The null hypotheses investigated were that *L. variegatus* would not uptake, accumulate or depurate ZnO NPs and bulk particles after a 48 hour uptake and 48 hour depuration period. A further aim was to assess the impact of organic matter on the uptake, accumulation and depuration of ZnO NPs and bulk particles in *L. variegatus*. The null hypothesis investigated in this part of the study was that organic matter would have no effect on the uptake, accumulation and depuration of ZnO NPs and bulk particles in *L. variegatus*.

6.2 Materials and methods

These studies were carried out at Heriot Watt University.

6.2.1 Study Preparation

The medium used in these studies was as described in Chapter 3, Section 3.2.1; worms were maintained as described in Chapter 3, Section 3.2.2; equipment was maintained as described in Chapter 3, Section 3.2.3; and worms were physiologically synchronised prior to each experiment as described in Chapter 2, Section 2.2.2.

6.2.2 Study 1: Initial protocol

All glass used was acid washed prior to the experiment according to chapter 3, section 3.2.3. Worms were physiologically synchronised twelve days before testing. Stock solutions (0.5mg/ml) of ZnO NPs and bulk particles were prepared in EPA HW medium and sonicated for 30 minutes. After sonication dilutions of 2.5 and 10mg/L ZnO were prepared in EPA HW medium and EPA HW medium with 5mg/L HA. After the twelve days synchronisation, three worms were blotted dried, wet weighed to get an average wet weight, followed by 24 hours in an oven at 60˚C and reweighing to get an average dry weight. Following this, one worm was added into a glass vial containing 20ml of exposure concentration. At 0 hours, three vials, from each concentration including the control, were selected at random and worms were removed and transferred into a glass vial containing 1ml deionised (DI) water and 500µl 70% HNO_3. At 1 hour, 3 vials were chosen at random and worms were individually transferred with a pastette onto a glass petri dish cover. A metal pick was used to pick up the worm (to ensure no liquid was transferred) and place it into another glass vial containing 5ml 10µM EDTA for five minutes. After this the worm was transferred with a pastette onto a clean glass petri dish cover and a clean metal pick was used to transfer it to a clean vial containing 1ml DI water and 500µl HNO_3. All three worms were combined for further analysis. Samples were combined due to cost constraints and time constraints on the equipment. A 5ml sample of the EDTA solution was kept for analysis and 5ml of the exposure solution was also kept for analysis. The exposure vials were rinsed in the EDTA solution for 5 minutes and this was also kept for analysis. This procedure was repeated at 4, 8, 24 and 48 hours. After 48 hours all remaining worms were transferred into clean EPA HW medium and allowed to depurate over 0, 1, 4, 8, 24 and 48 hours. The worms and exposure suspensions were treated the same way as they were previously for the uptake portion of the study.

Once all of the samples had been collected the samples were digested in 70% HNO_3. Worm samples were preserved in HNO_3 and so were vortexed to ensure the worm tissues were dissolved and no longer visible. All other samples were digested in 2ml HNO_3 for 24 hours. After the 24 hour period the worm samples were made up to 5ml, all other samples were made up to 20ml using distilled water and were placed into a water bath at 60˚C for 24 hours. All samples were then filtered through a GF/C filter.

Worm samples were made up to 5ml and all other samples were made up to 20ml with distilled water (if necessary). Samples were then measured by inductively coupled plasma atomic absorption spectrophotometry (ICP-AAS). If necessary the samples were diluted to ensure they could be read by the ICP-AAS. Standards were prepared for the ICP-AAS at 0, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10ppm Zn.

6.2.4 Study 2: Improving initial protocol as described in section 6.2.3

As a result of the data obtained from section 6.2.3 it was decided that the protocol described in section 6.2.3 needed to be improved. A stock solution (0.5mg/ml) of ZnO NPs in EPA HW medium was made up and sonicated in a sonication bath (Ultrawave Q series, 400W) for 30 minutes. Following sonication, a 10mg/L dilution of ZnO NPs in EPA HW medium was made from the stock and 10ml per vial ZnO NP of this suspension was added to thirty disposable plastic vials. Plastic disposable vials were used to avoid contamination from inadequate rinsing of used glass vials. To digest each sample 1ml 70% HNO_3 was added to each vial for 24 hours at room temperature, followed by 24 hours at 60˚C in a water bath. The vials were divided into five groups with six vials per group (see Table 6.1). Each set of six vials were filtered through a GF/C filter but the filter holder was rinsed in a different solution for each one, according to table 6.1 below. Following the rinse of the filter 10ml of DI water was passed through a new GF/C filter into a new plastic vial. This solution was run through the ICP-AAS. If necessary the samples were diluted. The standards were prepared as described previously in section 6.2.3.

Table 6.1: The rinses used on the GF/C filter holder

Group	**1st Rinse**	**2nd Rinse**
1	DI water	DI water
2	0.1M HNO_3	DI water
3	0.1M EDTA	DI water
4	0.1M HNO_3 + 0.1M EDTA	DI water
5	No rinse	No rinse

6.2.4 Study 3: Improved protocol

Based on the results obtained from Section 6.2.4 a new protocol was developed. The study was run as described in Section 6.2.3 however a number of changes were made. Plastic disposable vials were used for the entire study as opposed to acid washed glass vials, to reduce potential contamination from inadequately rinsed glass vials. All worms were wet weighed prior to testing as dry weighing of these worms was not possible due to the size of the study and the potential for the worms to desiccate. It was decided to reduce the size of the study because of time and cost constraints. The size of the study was reduced to a comparison of controls against 10mg/L ZnO NP and bulk particle solutions and by only running the worm samples on the AAS and not the exposure solution, glassware rinses and EDTA worm rinses. The final change was the rinsing of the filter holder. Based on the results obtained in section 6.2.4 it was decided that the filter holder should be rinsed in 0.1M HNO_3 followed by a rinse in DI water.

6.3 Results

6.3.1 Study 1: Initial protocol

This study was not successful as the data were found to be highly variable which suggested that there was a high level of contamination in the study. As a result of this, the protocol was investigated and improved upon.

6.3.2 Study 2: Improving initial protocol

Data were tested for normality using a Kolmogorov Smirnov test and found to be normally distributed ($Z = 0.604$, $P > 0.05$). A one way ANOVA was performed to assess if there were any significant differences in the amount of zinc remaining in the DI water filtrate after the filter holder had been rinsed in various solutions. The one way ANOVA result (Table 5.1) suggested there was a significant difference between at least 2 types of rinse ($F = 4.293$, d.f. = 4, $P < 0.05$). Tukey tests were performed in order to see where these differences lay. It was found that the only significant difference was found to be between the nitric acid rinse and no rinse at all (Figure 6.1). The nitric acid rinse removed significantly more Zn from the filter holder and so the

protocol for the worm study was adjusted to include a nitric acid rinse and DI water rinse of the filter holder.

Table 6.2: The output for the ANOVA investigating the effects of rinsing the GF/C filter holder in five different rinses.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.000089	4	.000232	4.293	0.011
Within Groups	0.000104	20	.000005		
Total	0.000194	24			

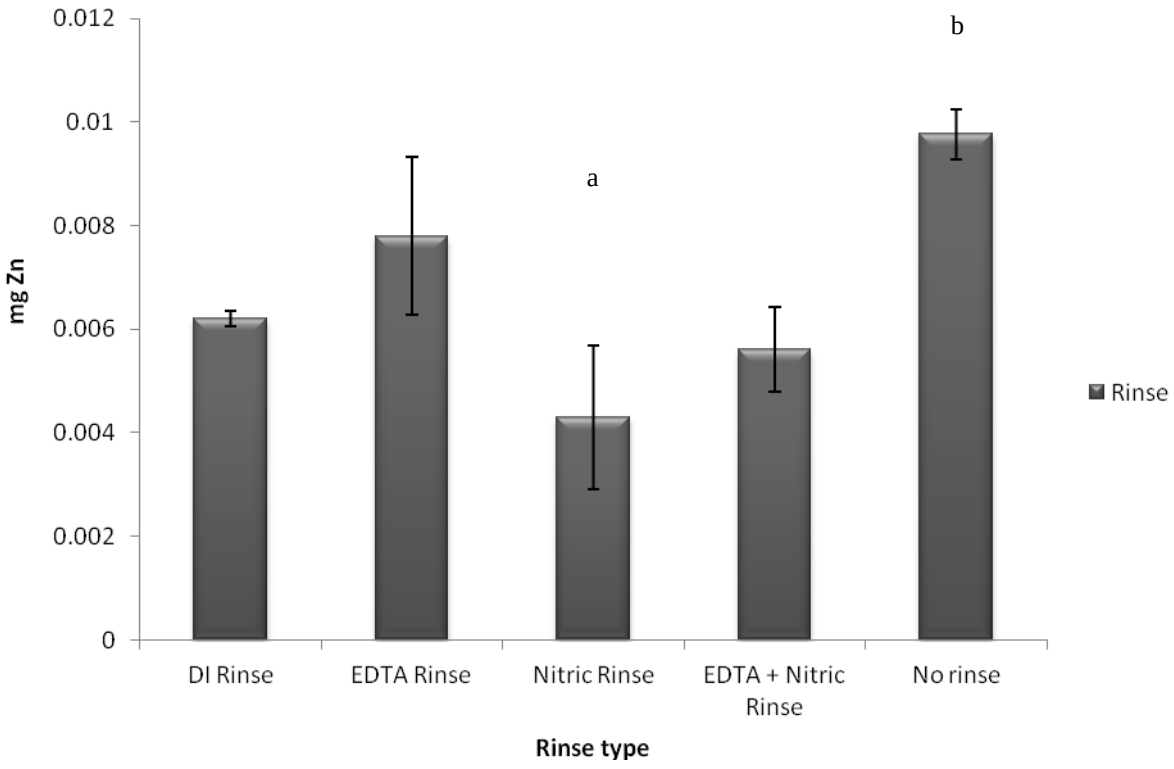

Figure 6.1: Amount of Zn in each filtered sample after a variety of filter holder rinses (data represents means ± SE; a different letter indicates a significant difference i.e. a is significantly different from b).

6.3.3 Study 3: Improved protocol

Data were tested for normality using a Kolmogorov Smirnov test and were found to be parametric ($Z = 0.742$, $P > 0.05$). One way ANOVA tests were performed on the uptake and release data to assess if there were any significant differences in the amount of zinc contained in worms exposed to EPA HW medium alone, 10mg/L ZnO NP in EPA HW medium suspensions and 10mg/L bulk ZnO particles in EPA HW medium suspensions. For the uptake study there was no significant difference found between the control, NP and bulk worms ($F = 0.948$, d.f. = 2, $P > 0.05$) (Table 6.3).

Table 6.3: ANOVA output investigating the uptake of ZnO NPs and bulk particles after a 48 exposure.

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.039	2	0.020	0.948	0.403
Within Groups	0.432	21	0.021		
Total	0.471	23			

For the release study the ANOVA result suggested that there was a significant difference between at least two exposures ($F = 10.851$, d.f. = 2, $P < 0.001$) (Table 3). Tukey tests were performed to find out where these significances lay. Worms exposed to ZnO NPs over 48 hours and allowed to depurate for 48 hours were found to have significantly more Zn in them than worms exposed to EPA HW medium alone ($P < 0.01$) and worms exposed to bulk ZnO particles ($P < 0.001$) (Figure 6.2).

Table 6.4: ANOVA output investigating the depuration of ZnO NPs and bulk particles after a 48 hour exposure.

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.283	2	0.141	10.851	0.001
Within Groups	0.260	20	0.013		
Total	0.543	22			

However, the data obtained in this study were highly variable as each data point was an individual worm and different worms were used for the uptake part of the experiment and the release part of the experiment, due to the nature of the study. Ten replicates of each concentration were run but a few worms died during testing (as has been seen in other studies), so between 6 and 9 data points were used for statistical analyses. A power test was used to determine how many worms would be necessary to use in order to iron out the variability. This power test indicated that large numbers of worms would be needed in order to address this point (Table 6.5).

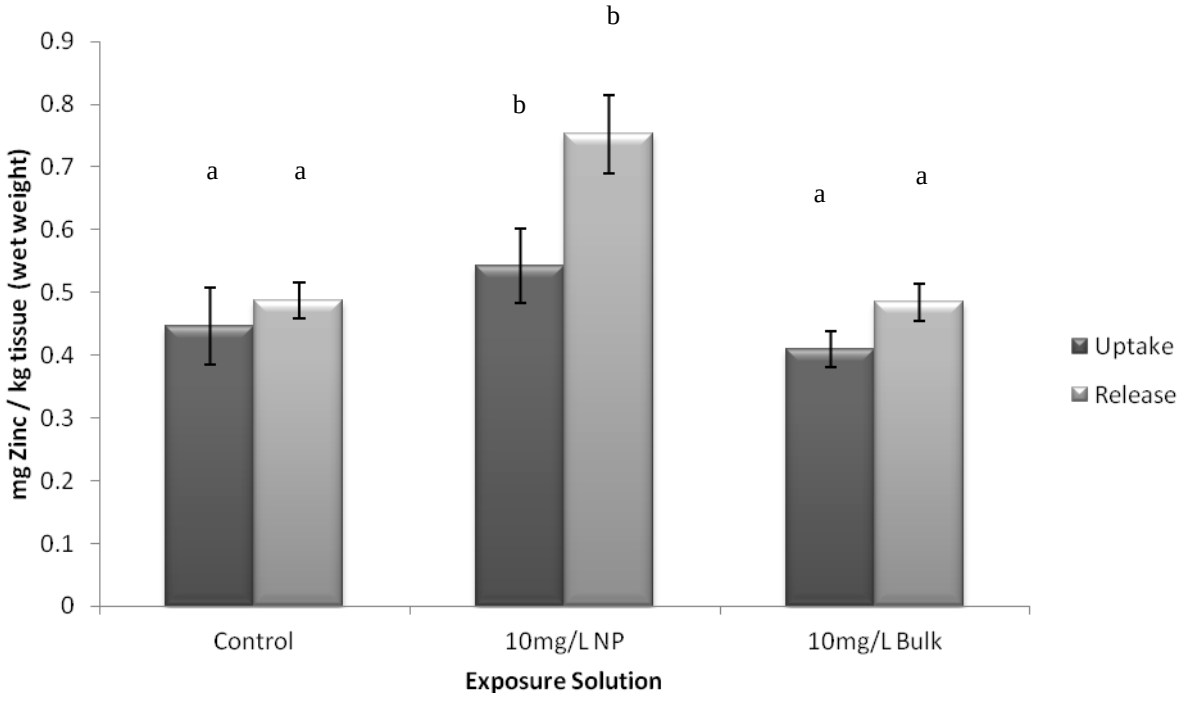

Figure 6.2: Amount of Zn per kg of worm tissue after 48 hours of uptake and 48 hours of depuration (mean ± S.E; a different letter indicates a significant difference i.e. a is significantly different from b).

Table 6.5: The number of worms (calculated using a power test) needed to reduce the variability of the study.

Uptake		**Release**	
Control vs. NP	48 worms	Control vs. NP	7 worms
Control vs. Bulk	235 worms	Control vs. Bulk	13221 worms
NP vs. Bulk	13 worms	NP vs. Bulk	7 worms

Since so many worms would be needed, and the potential for contamination in this study, it may not be a suitable protocol for use with zinc as zinc is present widely in the environment.

6.4 Discussion

6.4.1 Initial protocol

This protocol was not successful as there was a high level of variability in the results obtained from the ICP-AAS, most likely as a result of contamination. This contamination may have been introduced into the study in a number of routes. Firstly, there may have been a background level of zinc in the worm themselves from their food and culture. Secondly, it was not possible to use disposable filter paper holders due to the size of the study and the cost involved. For these reasons it was concluded that this protocol was not suitable for assessing the uptake, bioaccumulation and depuration of ZnO NPs or bulk particles and so an investigation was performed into improving the protocol for future studies.

6.4.2 Improving initial protocol

In order to reduce the contamination seen in the first study a number of changes were made to the original protocol. As it was too expensive to run the study using disposable filter holders an investigation into a variety of rinses was performed in order to ascertain whether: i) rinsing the holder could cause significantly less contamination than no rinse

at all, and ii) which rinse was best. No rinse was successful in completely removing all contamination, however the nitric acid rinse followed by a DI water rinse was found to have significantly less contamination than no rinse at all. It was decided that the study would proceed with the following improvements: i) all equipment (where possible, e.g. vials, petri dishes, picks, new gloves for each step) used were disposable, ii) the study size was reduced (due to the cost of using disposable equipment) and iii) the filter holder was rinsed in 0.1M nitric acid and DI water following each sample.

6.4.3 Improved protocol

Handy *et al*. (2008) suggested that it may be possible to measure metal NP concentrations in the tissue of organisms by a method which uses inductively coupled plasma mass spectrophotometry (ICP-MS), or a similar technique (e.g. ICP-AAS), and which includes an acid digestion step. This approach was said to be good for materials that are not naturally present in the organisms (e.g. gold) but possibly not as effective in picking up the NP metal effect from background levels already in the organism (Handy *et al*., 2008). The present study was found to have a high level of variability and it was decided that while this protocol may be appropriate for metals that are not naturally present in relatively large quantities in the environment, it was not suitable for investigating the uptake, bioaccumulation and depuration of ZnO NPs and bulk particles. Dawson *et al*. (2003) investigated the accumulation of bulk Zn in *L. variegatus* and found that the background levels of Zn in the worm may have affected the result and that variability in the measurements was very high, using ICP-AAS. In order to improve the protocol it would be advisable to use all disposable equipment (e.g. vials, petri dishes, picks, filter holders, gloves etc.) where possible, to ensure that the medium used is prepared with minimal/no contamination and also to allow the worms to fully purge their guts after physiological synchronisation and before testing. These steps, however, would significantly increase the cost of the study and also the time required to conduct it.

6.5 Summary of this study

The uptake, accumulation and depuration of ZnO NPs and bulk particles (with and without 5mg/L HA) in *L. variegatus* over a 48 hour period of uptake and a 48 hour period of depuration were investigated in this study. The protocol used was not successful as a result of contamination of zinc and would require significant reduction in contamination in order for this protocol to be considered successful. This protocol may be used if time, money and space are not an issue however this is rarely the case. Other avenues of investigation that may provide a better understanding of the uptake and depuration of ZnO in *L. variegatus* could include the use of dyes such as Newport Green PDX™ Acetoxymethyl Ether, FluorZin™-3, AM, cell permeant and RhodZin™-3, AM, cell permeant.

Chapter 7 Histological studies: The investigation of the effects of ZnO nanoparticles and ZnO bulk particles on the anatomical structure of *Lumbriculus variegatus.*

7.1 Introduction

Histology is the investigation of the microscopic structure of cells, tissues and organs (Van Lommel, 2003) and can provide useful information on the effects of contaminants in aquatic species.

7.1.1 The use of histology in toxicological studies concerning aquatic organisms

A number of studies have employed histological techniques to investigate the effects of numerous aquatic pollutants. Several studies have investigated the histological impact of heavy metals in aquatic species. Chen *et al*. (2012) found that copper induced gill and liver injury in yellow catfish (*Peltepbagrus fulvidraco*) using histological techniques. Further studies investigated the effect of heavy metals on Asian green mussels (*Perna viridis*) (Vasanthi *et al.*, 2012) and on the golden apple snail (*Pomacea canaliculata*) (Dummee *et al*., 2012). Histological damage was seen in the gills, digestive organs and in the case of the mussel, in the adductor muscle. Zinc has been investigated histologically using numerous species such as yellow tail labara (*Astyanas* aff. *bimaculatus*), white shrimp (*Litopenaeus vannamei*), gobies (*Synechogobius hasta*) and tilapia (*Oreochromis mossambicus*). Zn was found to cause profound gill changes in yellow tailed lambari (dos Santos *et al.*, 2012), alterations in the hepatopancreas of white shrimp (Wu *et al*., 2008), impaired histological structure of the gills and spleen in gobies (Zheng *et al.*, 2011) and significant histological changes in the liver of tilapia (Van Dyk *et al*., 2007). Other contaminants such as UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), *N*-acetyl-hexozaminidese (HEX), acid phosphatase (AcP), β-glucuranidase (β-GUS), perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) have also been investigated using histological techniques. Christen *et al*. (2011) investigated the effects of EHMC in fathead minnows (*Pimphales promelas*) and found that the testes and ovaries of the fish showed significant histological changes.

Raftopoulo and Dimitriadis (2012) investigated the effects of HEX, AcP and β-GUS and found that all caused structural changes in the epithelium of large mussels (*Mytlius galloprovincialis*). Finally, Keiter *et al*. (2012) described changes in the histological structure of liver tissue in zebra fish exposed to PFOS and BPA.

7.1.2 The use of histology in nanoparticle aquatic toxicology studies

The toxic effects of a number of NPs have been investigated using histological techniques. TiO_2 NPs have been shown to cause histological damage in carp (*Cyprinus carpio*), zebra fish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*). Hao *et al*. (2009) reported that TiO_2 NPs caused gill and liver damage in carp, Ramsden *et al*. (2013) reported that TiO_2 NPs caused no histological damage in zebra fish (however reproduction was affected) and Boyle *et al*. (2013) reported that TiO_2 NPs caused morphological changes in brain blood vessels of rainbow trout. Diamond NPs have been shown to accumulate within the digestive tract and adhere to the exoskeleton of *Daphnia magna* using histological techniques (Mendonça *et al*., 2011). The authors suggested that this could lead to potential food absorption issues for the water flea. Cu NPs were shown to cause histological damage in the intestine, liver, brain, gill and muscle of rainbow trout (Al-Bairuty *et al*., 2013). Ag NPs have been shown to cause damage to the gills of Atlantic salmon (*Salmo salar*) using histology (Farmen *et al*., 2012) and finally, Fe NPs have been shown to cause structural damage to the gills and intestinal tissues of medaka (*Oryzias latipes*) (Li *et al*., 2009).

7.1.3 Lumbriculus variegatus and histology

Very few papers have been published to date which have used histology to investigate the anatomy of *L. variegatus*. Lesiuk and Drewes (2001) used histology to investigate the regeneration of body segments in *L. variegatus* after transection and ablation. They fixed the worms in a glutaraldehyde and cacodylate solution and stained the sections with toluidine blue. Martinez *et al*. (2006) investigated the effects of boric acid on the anatomy of *L. variegatus* using histological techniques by fixing the worms in formaldehyde and staining the tissues with toluidine blue. More recently, Sardo *et al*. (2011) investigated the effects of lead on the anatomy of *L. variegatus* after a 10 day exposure. They fixed the tissues in Bouin's solution and stained them in haematoxylin

and eosin (H and E) stain. After 10 days they noted that no change was visible in the histological sections. The present study was designed to investigate the anatomy of *L. variegatus* after exposure to ZnO NP and bulk particles.

7.1.4 Fixation and staining of tissues

One of the most important aspects of histology is the immediate preservation of the structure of the tissue (Méndez-Vilas & Díaz, 2010). An ideal fixative would preserve the original structure of the tissue and should allow the tissue to be as close as possible to its natural state (Boeck, 1989, in Meyer and Hornickel, 2010). During this study three fixatives were investigated: formaldehyde, Bouin's solution and Davidson's solution. The use of formaldehyde was introduced by Friedrich Blum in 1893 (Meyer and Hornickel, 2010). He demonstrated that formaldehyde affects the solubility of proteins by the formation of methylene compounds with amino, amide and hydroxyl groups. The penetration of these chemicals is rapid but occurs at different rates (Boeck, 1989, in Meyer and Hornickel, 2010; Buesa, 2008). This fixation method also alters the pH of the tissue. Both penetration and pH of the tissue can affect the structure of the tissue and increase or decrease the number of binding sites for staining (Hayat, 1993). Bouin's solution was introduced by Pol André Bouin in 1897 and it contains formaldehyde, piric acid and acetic acid (Meyer and Hornickel, 2010). This fixative penetrates more rapidly and evenly and is less damaging to protein structures than formaldehyde alone (Lillie and Fulmar, 1976; Pearse, 1985; James and Tas, 1984), however the addition of piric acid causes the solution to be hazardous. Davidson's solution was named after William McKay Davidson but was first published by Moore *et al*. in 1953. Davidson's solution is similar to Bouin's solution however the piric acid is replaced with alcohol. This replacement allows for rapid penetration of the fixative however the tissues can shrink as a result due to dehydration of the tissue (Latandresse *et al*., 2002). After fixing and sectioning, the tissues must be stained in order to view the structure of the tissue. Fixation methods can affect the physicochemical state of tissues and so staining methods may have different results depending on the fixation technique used (Hayat, 1993). In this study a H and E stain was applied to the sections. H and E stain is the most frequently used staining method in anatomical pathology worldwide (Cook, 1997). Hematoxylin was first introduced as a stain by Böhmer in

1865 and eosin was introduced by Fischer in 1875. The method of combining these stains was developed by Wissowzky in 1876 (Gill, 2009). Hematoxylin stains acid structures, such as the nucleus, blue, while eosin stains basic structures, such as the cytoplasm, pink or red (Young *et al*., 2006).

7.1.5 Aims

The aim of this study was to investigate the uptake by and effects of ZnO NPs and bulk particles on the histological structure of *L. variegatus*. The null hypothesis investigated was that ZnO NPs and bulk particles would not be taken up by and would not have any effect on the histological structure of *L. variegatus*. In this context a further aim was to assess which fixative was the most suitable for use when preserving *L. variegatus*.

7.2 Materials and methods:

Exposure studies were carried out at Heriot Watt University (HWU) and histological studies were carried out at Edinburgh Napier University and HWU. Worms were exposed to 0, 1.25, 2.5, 5 and 10mg/L ZnO NPs and bulk particles for 96 hours as described in chapter 3, section 3.2.6. For the Bouin's solution study worms were also exposed to positive controls of 0.2µM and 0.4µM $CuSO_4$, 2.5mg/L and 10mg/L $ZnSO_4$, 20mg/L carbon black (CB) NPs, 10mg/L, 50mg/L, 75mg/L and 100mg/L TiO_2 NPs (20nm) and 50mg/L and 100mg/L TiO_2 (250nm) bulk particles. Multiple particle types were investigated in order to act as a comparison to the worm sections exposed to ZnO NPs and bulk particles. These concentrations were chosen as another PhD student, Simon Little, ran these tests in order to assess behavioural toxicity as a result of exposure to these particles. Worms from Mr. Little's studies were then used for my histological studies.

7.2.1 Bouin's solution studies

Following exposure the worms were preserved in Bouin's solution for at least 5 hours before being transferred into 70% ethanol for longer term storage and transfer from HWUU to Edinburgh Napier University. The twelve pots of a Leica processor were

filled in the following order; 70%, 80%, 90% industrial methylated spirit (IMS) alcohol, three pots of absolute ethanol, three pots of 100% xylene and two pots of surgipath tissue embedding wax pellets, formula "R". Worms were removed from the 70% ethanol and placed into biopsy bags which were then stapled shut. The stapled bag was placed into a pencil labelled cassette and snapped shut. Sealed cassettes were immersed in 70% ethanol to avoid the worm tissues drying out while other cassettes were prepared. When ready for processing all cassettes were placed inside a metal basket attached to pot number one. The tissue programme was set for 90 minutes per pot and allowed to run overnight.

A number of hours prior to removing the cassettes from the processor a Leica EG1160 paraffin embedding centre was switched on to allow the wax to melt. Formula "R" wax pellets (Surgipath), heated to 60 - 70˚C, were used. Once the worms were processed the cassettes were removed from the processor and transferred to the heated cassette bath of the embedding centre. Biopsy bags were removed from each cassette and placed onto the hot plate (60 - 70˚C) of the embedding centre in order to melt off any excess wax. Scissors were used to cut open the bags and worms were carefully removed onto the hot plate. Worms were sectioned into approximately 5mm lengths using a scalpel. Heated metal moulds were removed from the mould heating bath of the embedding station and filled with a small amount of molten wax. This wax was cooled slightly using the cold plate of the embedding station and worms were positioned into the wax, as required, immediately. The number cassette which corresponds to the tissue was then snapped into place on the mould and more molten wax was added. The mould was tapped gently to ensure no bubbles remained within the wax. The mould was placed onto the cold plate (-5˚C) for approximately 30 minutes to allow the wax to set. Once it had cooled the wax block (attached to the cassette) was levered out of the metal mould ready to be cut on the microtome.

A Leica RM2125RT microtome, set to cut at 5µm, was used to section the worm tissues. The wax block was attached to the clamp of the microtome. A disposable blade was inserted into the blade holder and clamped shut. The wax block was trimmed until the worm tissue was exposed on the surface prior to sectioning. After trimming, the block was placed face down on the cold plate for at least 5 minutes. The cooled block was then reattached to the microtome cassette holder and cut into thin ribbons of wax. Ribbons were removed from the microtome using a forceps and carefully placed

into a water bath (45 – 50 ˚C). An "X-tra Adhesive" glass slide, placed underneath the wax section, was used to pick up the section from the water. The slide with wax was allowed to air dry for at least 6 hours.

Once dry, slides were placed inside a metal rack and immersed in xylene for 5 minutes to remove the wax. The xylene was replaced with alcohol immersing the slide for 3 minutes in 100%, 95%, 80% and 70% IMS alcohol. Following this, slides were immersed in distilled water for 5 minutes. To stain the slides they were immersed in hematoxylin for 1 minute and rinsed in slow running tap water for 10 seconds, followed by immersion in distilled water for 1 minute. Slides were then immersed in eosin for 30 seconds, followed by 3 minutes in 95% IMS alcohol and 5 minutes in 100% IMS alcohol. Finally, the slides were immersed in xylene for 5 minutes. The stained tissue was identified on the slide and a drop of pertex mountant was placed on top to adhere a cover slip.

7.2.2 Formaldehyde studies

After exposure worms were fixed in formaldehyde (10% formaldehyde in saline) for 24 hours before being transferred directly into the processor at HWU. The twelve pots of the processor were filled in the following order; two pots of 70% ethanol, two pots of 80% ethanol, two pots of 90% ethanol, three pots of 100% ethanol, three pots of histoclear II and two pots of Surgipath tissue embedding wax pellets, formula "R". Worms were removed from the 10% formaldehyde solution and placed into biopsy bags which were then stapled shut. The stapled bag was placed into a pencil labelled cassette and snapped shut. Sealed cassettes were immersed in 70% ethanol to avoid the worm tissues drying out while other cassettes were prepared. When ready for processing all cassettes were placed inside a metal basket attached to pot number one. The tissue programme was set for 60 minutes per alcohol and histoclear II pot and 90 minutes per wax pot and was allowed to run overnight.

Following processing, the tissues were removed, cut and blocked in the same process as described in section 7.2.2. Once on glass slides the sections were rehydrated in histoclear II twice for 3 minutes, Histoclear 1:1 with 100% ethanol for 3 minutes, twice in 100% ethanol for 3 minutes, and finally three minutes in each of 95%, 70% and 50% ethanol (respectively). Slides were stained by immersing them in hematoxylin for

1 minute, followed by a ten second immersion in distilled water. The stain was allowed to develop under a slow running tap for 5 minutes. The slides were then immersed in distilled water for a further minute. Following this, slides were immersed in eosin for 30 seconds, followed by 3 minutes in 95% IMS alcohol and 5 minutes in 100% IMS alcohol. The slides were then immersed in histoclear II for 5 minutes. The stain tissue was identified and a drop of pertex mountant was used to mount a cover slip.

7.2.3 Davidson's solution studies

After exposure worms were fixed in Davidson's solution (20 parts 38% formalin, 10 parts glycerol, 10 parts glacial acetic acid, 30 parts absolute ethanol and 30 parts distilled water) for 24 hours before being transferred directly into the processor at HWU. The processing, embedding, staining and sectioning of these worm tissues were conducted as described in Section 7.2.2.

7.2.4 Imaging slides

Slides were imaged using an inverted microscope at HWU, using a Canon SLR EOS 600 camera. Photos were downloaded from the camera using Windows Photo Viewer and Image J was used to scale the images.

7.2.5 Criteria for assessing morphological change within the worm

The criteria used to assess whether any damage occurred in the worm compared to the control can be found in table 7.1.

Table 7.1: Criteria for assessing morphological changes after exposure to ZnO NPs or ZnO bulk particles.

Structure:	**Damage?**
Body muscle	Has the muscled thinned/thickened compared to the control?
Gut muscle	Has the muscled thinned/thickened compared to the control?
Shape of the gut	Has the gut shape changed compared to the control?
Epithelial cells	Have the epithelial cells thinned/thickened compared to the control?
Gut epithelial cells	Have the gut epithelial cells thinned/thickened compared to the control?

7.3 Results:

7.3.1 Condition of the worms after fixation

In each of the three fixatives the worms were found to have very different textures, i.e. their colouring was different, their size was different and their malleability was different. Worms removed from Bouin's solution were found to be stiff and brittle and were coloured yellow (Figure 7.1a). Worms removed from formaldehyde were also coloured yellow, were softer and more malleable but they curled onto themselves making them difficult before embedding (Figure 7.1b). Finally, worms removed from Davidson's solution were found to be very soft, shrunken, almost jelly like and were often transparent (Figure 7.1c). It was difficult to section the worms fixed in Davidson's solution as it was not possible to see the tissues within the wax.

Figure 7.1: Worms after fixation in Bouin's solution (a), formaldehyde (b) and Davidson's solution (c).

7.3.2 Bouin's solution studies

Of the images taken during this study 70% were suitable for further analysis. The remaining 30% were not useful due to artefacts caused by the sectioning process (i.e. the tissue samples were dragged when sliced on the microtome). Figure 7.2 (images a and b) shows the structure of a control worm. It was not possible to observe any ZnO NP or bulk particles within the worms (Figures 7.3 and 7.4 respectively). It was also not possible to determine whether there was any damage caused by the bulk or NP ZnO to the structure of the worm.

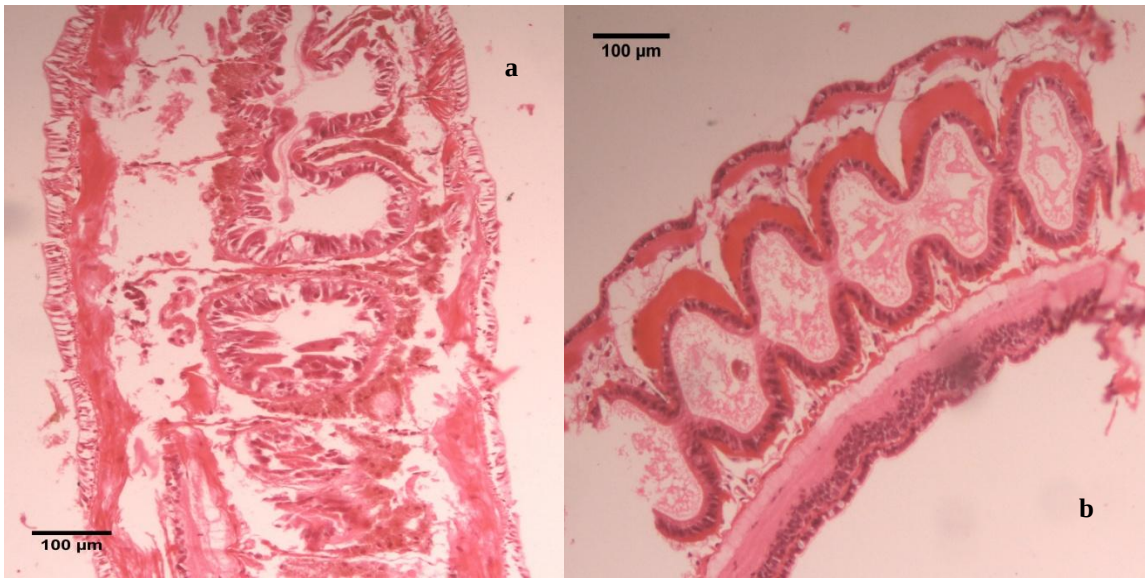

Figure 7.2: Histological image of control worms after 96 hours in EPA HW medium (a) and EPA HW medium with 5mg/L HA (b).

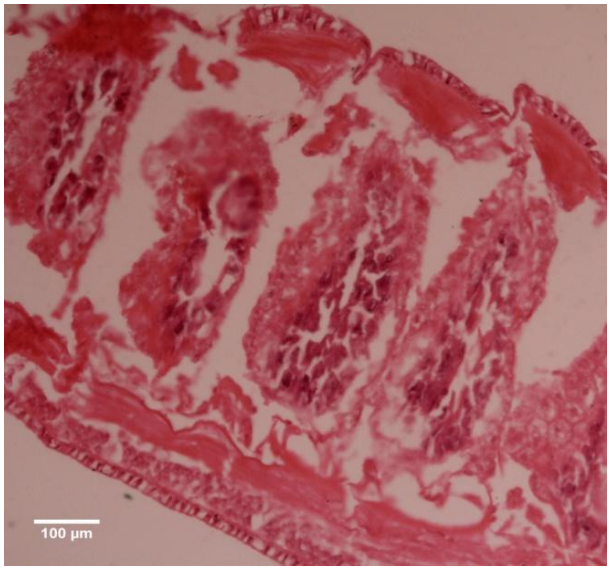

Figure 7.3: Histological image after 96 hours of a worm exposed to 1.25mg/L ZnO NP with 5mg/L HA.

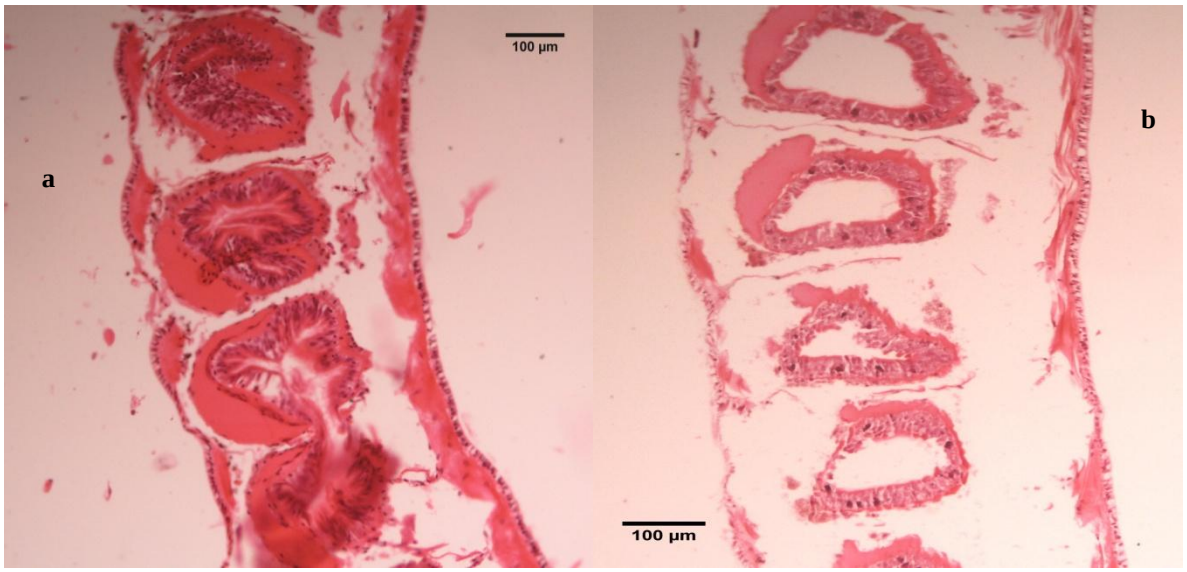

Figure 7.4: Histological image of a worm exposed to 5mg/L bulk ZnO particles (a) and 5mg/L bulk ZnO particles with 5mg/L HA (b) after 96 hours.

It was also not possible to see any damage caused by $ZnSO_4$ and $CuSO_4$ after a 96 hour exposure (Figures 7.5 and 7.6, respectively).

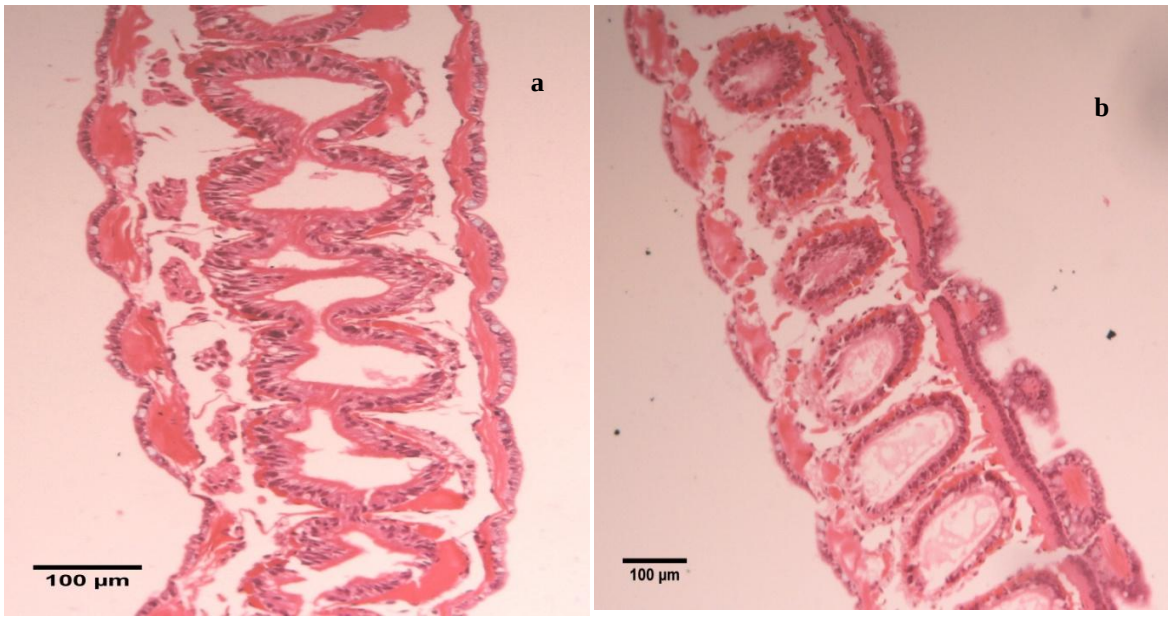

Figure 7.5: Histological image of a worm exposed to 2.5mg/L $ZnSO_4$ (a) and 2.5mg/L $ZnSO_4$ with 5mg/L HA (b) after 96 hours.

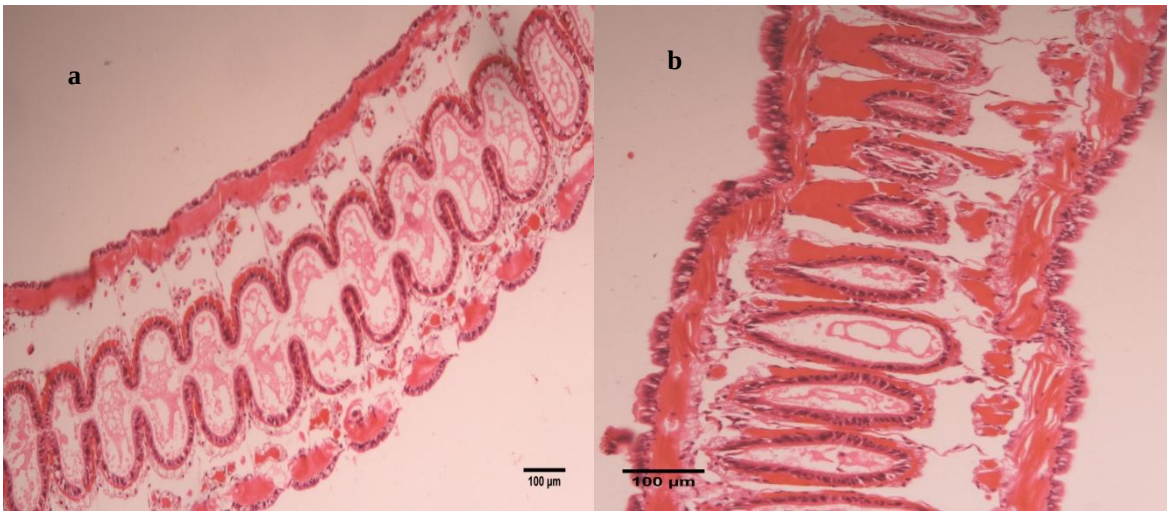

Figure 7.6: Histological image of a worm exposed to 0.2 µM $CuSO_4$ (a) and 0.2 µM $CuSO_4$ with 5mg/L HA (b) after 96 hours.

In the worms exposed to CB NPs it is possible to see small black specks within the gut of the worm (Figure 7.7a) which are not evident in any other exposure. It was not possible to see any TiO_2 particles within the gut of worms exposed to TiO_2 NPs and bulk particles (Figure 7.7b). It was not possible to see any damage in worms exposed to CB or TiO_2.

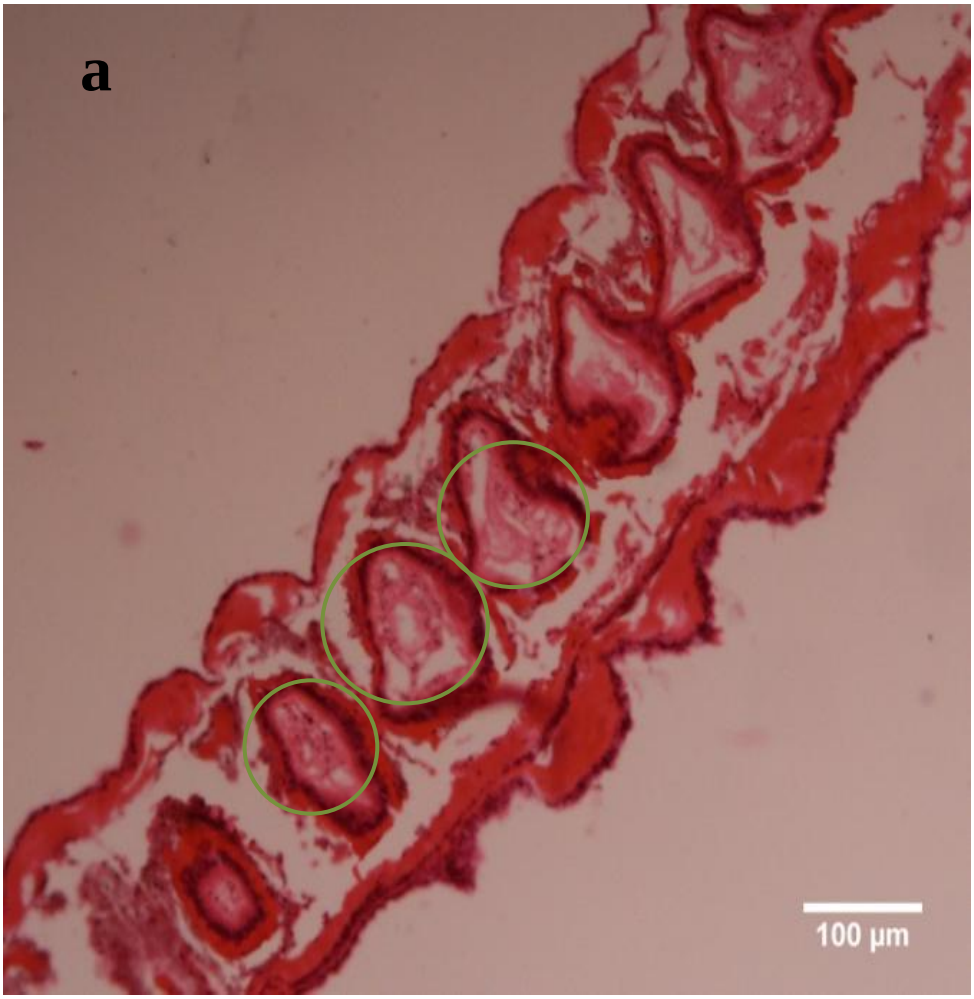

Figure 7.7: Histological image of a worm exposed to 20mg/L carbon black (CB) NPs (a) after 96 hours. The green circles show what appears to be CB NPs inside the gut of the worm.

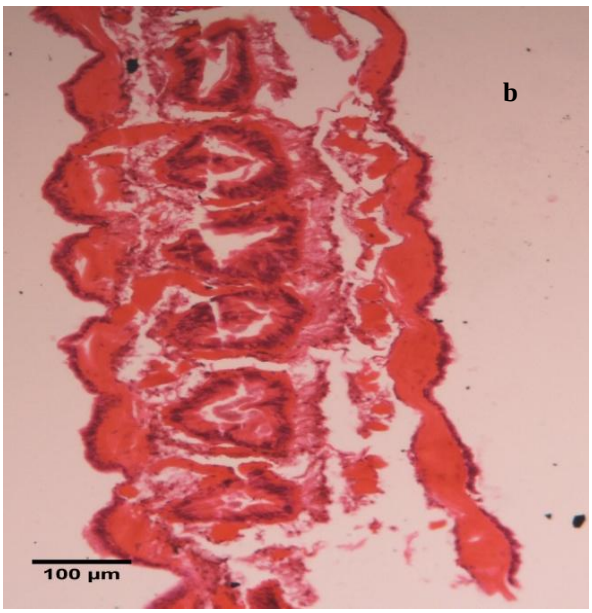

Figure 7.7b: Histological image of a worm exposed to and 50mg/L TiO_2 bulk particles (b) after 96 hours.

7.3.3 Formaldehyde studies and Davidson's solution studies

Within the formaldehyde study only 28% of the images taken were useful. Much of the worm tissues were skewed during sectioning. This may have resulted from the fixative or the sectioning technique. Examples of the skewing of tissues can be seen in figure 8. Within the Davidson's solution study 90% of the images were useful however sectioning was far more difficult and time consuming with this fixative than with either Bouin's solution or formaldehyde. An example of a worm fixed in Davidson's solution can be seen in Figure 7.9.

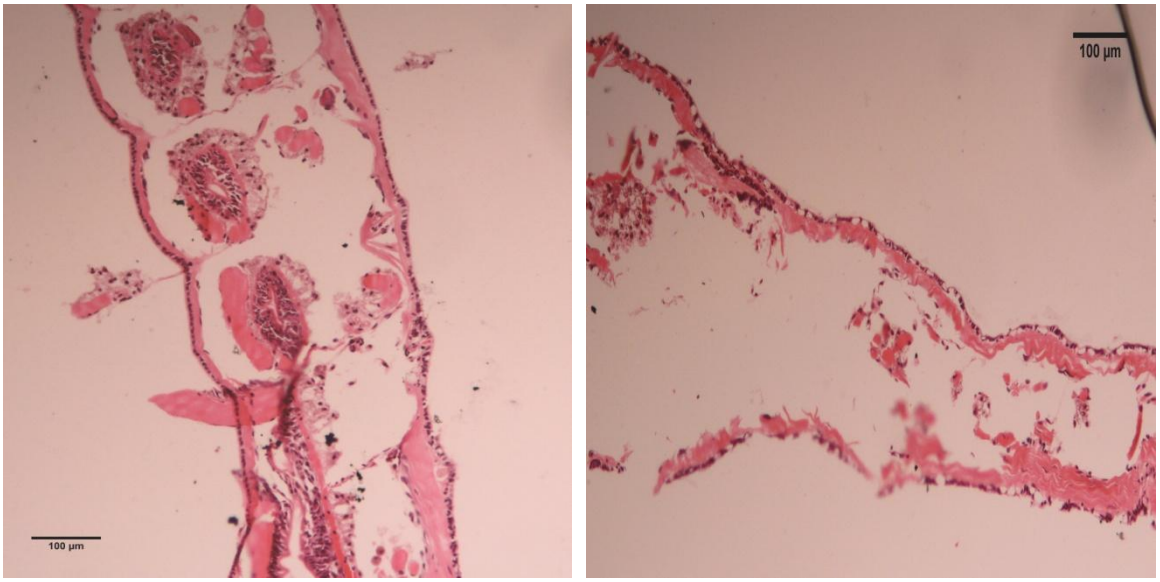

Figure 7.8: Histological images of worms preserved in formaldehyde. It is clear from these images that the tissues were damaged during sectioning and were not useful.

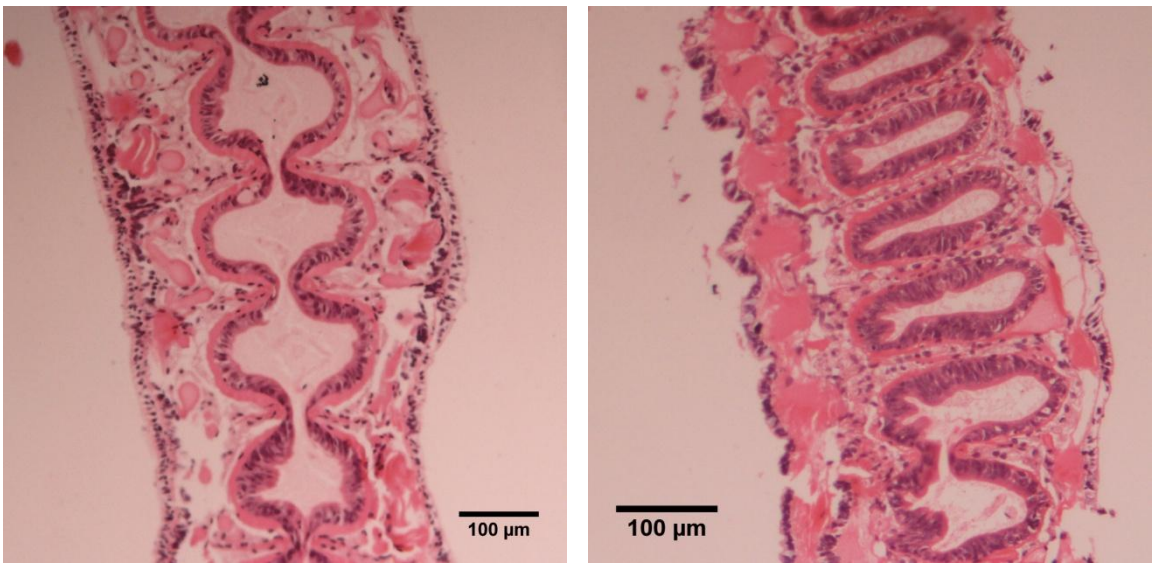

Figure 7.9: Histological image of worms fixed in Davidson's solution.

7.3.4 Comparison of fixatives

Based on the appearance and manoeuvrability of the worms after processing and the number of images that were suitable for use it was decided that best fixative for use with *L. variegatus* was Bouin's solution.

7.4 Discussion:

7.4.1 Comparison of fixatives

As mentioned in Section 7.1.2, the most important step in the histological process is fixing the tissue immediately after the end of a study. These fixatives were chosen as they were being used within the laboratory already and evidence had been found that the worms had been preserved in at least one (Bouin's) of them previously. The advantages of using Bouin's solution were that the tissues were coloured and easy to see in wax and they were easy to cut and manipulate during embedding, however the disadvantages included the tissues being brittle and also that a closed processor must be used due to the hazardous processing chemicals used. The other fixatives also had issues involved, such as with Davidson's solution the worms were soft and difficult to see in wax and with the formaldehyde the worms curled onto themselves making them hard to cut and manipulate during embedding, however the advantage of both Davidson's and Bouin's solutions was that they could be processed in an open machine. Based on the results from this study it was found that Bouin's solution was the most appropriate fixative when the time taken to process, embed, section and image the tissues were all taken into account.

7.4.2 Histology and Lumbriculus variegatus

A change in morphology of the anatomical structure of an organism after exposure to NPs can result in the impairment of functional capacity, recovery capacity or an increased susceptibility to harmful environmental conditions (Fadeel *et al.*, 2012). During this study no histological or morphological changes were identified in the anatomical structures of worms exposed to ZnO NPs, ZnO bulk particles, $CuSO_4$, $ZnSO_4$, TiO_2 NPs, TiO_2 bulk particles and CB NPs, using the described methodology.

As mentioned in the introduction, Sardo *et al*. (2011) stated that no change was visible in the structures of *L. variegatus* after a 10 day exposure to lead. It is possible that a 96 hour exposure is not a sufficient exposure time to induce visible changes in the worms. It was, however, possible to visualise black material in the gut of CB NP treated worms. *L. variegatus* have been shown to ingest particles (Chapter 2, Section 2.3.2) and so it is possible that these are agglomerated CB NPs, however this would need to be confirmed. It may be that the ZnO NPs were not visualised either because they had dissolved during exposure and/or as a result of their colour.

Future studies that involve investigating the effects of NPs on *L. variegatus* should include a longer exposure period (e.g. OECD 28 day sediment bioaccumulation study). This additional time may allow for any changes to occur within the tissues should a toxic effect be observed. It would also be interesting to use multiple stains in order to visualise a variety of components of the tissues. Other structures that may be stained include muscle and collagen, nerve tissue, enzyme activity in the gut, mucins and lipids. The van Gieson stain is used to stain connective tissue red, nuclei blue and cytoplasm yellow, silver stains are used to stain nerve tissue and other fine structures, acid and alkaline phosphatase stains are used to stain for enzyme activity, Alcian Blue is used to stain mucins and Oil Red O is used to stain lipids (Young *et al.*, 2006).

7.5 Summary of this study

Histological techniques were used in this study in order to assess any histological damage in *L. variegatus* as a result of acute exposure to ZnO NPs and bulk particles (with and without 5mg/L HA). It was found that Bouin's Solution was the best preservative to use with this tissue compared to Davidson's Solution and Formaldehyde. The histological technique employed in this study successfully indicated that there was no morphological damage incurred in the tissues of *L. variegatus* after a 96 hour exposure to ZnO NPs and bulk particles (with and without 5mg/L HA). This study indicates that histology is a useful and valid technique in assessing the toxic effects of ZnO NPs and bulk particles in *L. variegatus*.

Chapter 8 General Discussion

8.1 Summary of the findings in this thesis

This thesis was able to demonstrate that ZnO NPs can have toxic effects on *Lumbriculus variegatus*, however this toxicity is endpoint specific. While ZnO NPs induced both acute and chronic effects on the behaviour of *L. variegatus* they did not induce an acute oxidative response in the worms according to the GSH assay. The findings of this thesis have been summarised in Table 8.1 below.

Table 8.1: Summary of the findings of this thesis (x = "no", √ = "yes").

Chapter	Endpoint	Study length	Particle Type	HA	Endpoint Inhibition observed?	Toxic concentration	Protocol suitable for testing with *L. variegatus* & ZnO?
3	**Behaviour** Exposure in water	96 hours	NP	x	√	1.25-10mg/L	√
		96 hours	NP	√	x	-	√
		96 hours	Bulk	x	x	-	√
		96 hours	Bulk	√	√	5mg/L	√
5	**Behaviour** Exposure in paper "sediment"	28 days	NP	x	√	12.5-50mg/L	√
		28 days	NP	√	√	12.5-50mg/L	√
		28 days	Bulk	x	√	12.5-100mg/L	√
		28 days	Bulk	√	√	12.5-100mg/L	√
4	**Oxidative Stress** Exposure in water	0-96 hours	NP	x	x	-	√
		0-96 hours	NP	√	x	-	√
		0-96 hours	Bulk	x	x	-	√
		0-96 hours	Bulk	√	x	-	√
6	**Uptake** Exposure in water	0-48 hours	NP	x	-	-	X
		0-48 hours	NP	√	-	-	X
		0-48 hours	Bulk	x	-	-	X
		0-48 hours	Bulk	√	-	-	X
7	**Histology** Exposure in water	-	NP	x	x	-	√
		-	NP	√	x	-	√
		-	Bulk	x	x	-	√
		-	Bulk	√	x	-	√

After a 96 hour exposure ZnO NPs induced a negative behavioural response between 1.25 and 10mg/L, in contrast bulk ZnO particle did not. The addition of HA to the ZnO NPs prevented the impact of the particles on the behaviour of the worms, while the addition of HA to the bulk particles enhanced the effect on behaviour at 5mg/L (Table 8.1). An impairment of the escape behaviour in these worms would significantly impact their survival in the wild. Locomotor behaviours are integrally linked with the worm's ability to forage, sexually reproduce, avoid predators, disperse quickly and react to general environmental cues (Drewes, 1997). The difference between the NP and bulk result may be attributed to the difference in surface area between the two particle types. The BET study found that the surface area of the NPs was significantly greater than that of the ZnO bulk particles and the greater the surface area of a particle, the greater the reactivity of that particle and in turn the greater the potential for that particle to have an increased toxicity (e.g. Li *et al*., 2006; Pal *et al*., 2007; Navarro *et al*., 2008). The solubility of ZnO may have also played a role. The solubility of the NPs was found to be significantly greater than that of the bulk particles during the dialysis experiments. This would mean that the worms were exposed to more Zn ions in the NP exposures compared to the bulk particle exposures, giving rise to potential ionic toxicity. However, they may also have been exposed to insoluble NP/aggregates as the solubility equilibrium was reached at 0.9 ± 0.15 (SE) mg/L and the exposure concentrations in this experiment ranged from 1.25 to 10mg/L ZnO. This is something that is supported by a number of other studies (Heinlann *et al*., 2008; Xia *et al*., 2008; Blinova *et al*., 2010; Wong *et al.*, 2010). The dissolution of NPs may result in an additional layer of complexity when examining their potential toxicity (Xia *et al*., 2008). The species being used for testing is also an important consideration as the feeding behaviour of *L. variegatus* may have also played a role in the observed difference in toxicity between NP and bulk particles. The agglomerates that the worms may have come in contact with in the bulk study may potentially have been too large for them to ingest. Within the DLS study the hydrodynamic diameter of bulk ZnO particles were found to be significantly larger than ZnO NPs. The addition of humic acid mitigated the negative effect of the ZnO NPs. TEM images taken of ZnO NPs dispersed with 5mg/L HA indicated that the particles agglomerated and were also surrounded by what appeared to be an organic matrix. This matrix may have been what increased the stability of the dispersion (indicated by DLS data) and kept the agglomerates in suspension throughout

the exposure. Other studies have noted that humic acids increase the stability of NP suspensions (Chen and Elimelech, 2007; Handy *et al.*, 2008; Zhang *et al*., 2009). As the worms sat at the base of the vial during exposure there was less potential for them to have come into contact with the particles compared to NP exposures alone. The solubility (as determined in the dialysis experiment) of ZnO NPs in EPA HW medium and EPA HW with HA medium was not found to be significantly different so the worms in the HA exposures had the potential to come into contact with the same amount of Zn ions but potentially had less contact with ZnO NPs and agglomerates. At 5mg/L ZnO bulk with 5mg/L HA there was a significant negative effect on the behaviour of the worms. When examining the DLS data, HA does not appear to affect the hydrodynamic diameter of the bulk particles however their stability was increased with the addition of 5mg/L HA. It would be expected that since HA should have increased dispersion and kept the bulk particles in suspension, the worms would have come into less contact with the particles. However, it is possible that large agglomerates may have formed and deposited onto the area where the worm was placed. The TEM images produced at FENAC did appear to show agglomerates that looked different in EPA HW medium and EPA HW medium with HA.

Both ZnO NPs (12.5 – 50mg/L ± 5mg/L HA) and bulk particles (12.5 – 100mg/L ± 5mg/L HA) were found to have a significant negative effect on the behaviour of the worms after a 28 day paper "sediment" exposure (Table 8.1). When comparing NP toxicity to bulk toxicity, it was found that the NPs induced a greater negative effect on behaviour than the bulk particles. As discussed in the acute toxicity studies, the larger surface area (as determined by the BET study) and greater solubility (as determined by the dialysis study) of the ZnO NPs, compared to the ZnO bulk particles, may have a played a role in the differences in observed toxicity. HA did not affect the toxicity of either ZnO particle type. A number of studies have stated that natural organic matter (NOM) can increase the dispersion of NPs in aqueous media (silver NPs, Gao *et al*., 2012; carbon nanotubes, Chappell *et al*., 2009, carbon nanotubes, Lin and Xing, 2009), however Gao *et al*. (2012) also stated that at higher levels of NOM (i.e. >10mg/L) agglomeration and aggregation of silver NPs can increase. The combination of the added HA and food matter may have increased the NOM content of the test vessels to >10mg/L. If agglomeration and sedimentation of the particles were increased, the worms (which stayed at the bottom of the test vessel within

the paper "sediment") would have come in contact with the agglomerates which would not have been the case in the acute studies. The time factor may have also played a role in the different toxicity observed in the acute studies (96 hours) and the chronic studies (28 days). Finally, the introduction of food to this test may also have had an impact on the toxicity of the particles (± HA). The particles may have adsorbed onto the nettle leaf leading to agglomeration and sedimentation of particles. As mentioned previously behavioural changes in an organism can have significant ecological implications (Gerhardt, 1995) not only for the species themselves but also other non-benthic species that use the worms as a food source. It is likely that chronic exposure studies may give a clearer picture of the toxicity of ZnO and be more environmentally relevant than acute exposures (Hao *et al*., 2012). Detection of early warning signals such as behavioural changes in species is ecologically relevant and cheaper than chemical detection of contaminants within the tissues of the animal (Gerhardt, 1999).

Both ZnO NPs and bulk particles did not cause oxidative stress as measured by GSH depletion in *L. variegatus* after a 96 hour exposure to ZnO NPs and bulk particles (Table 8.1). A number of reasons may be put forward in order to potentially explain why an expected oxidative stress reaction was not observed. It has been suggested that pre-exposure to low intensity oxidative stress may enhance an organism's tolerance to a subsequent higher intensity oxidative stress (Lushchak, 2011). It is also possible that the ZnO exposure concentrations were too low to produce enough ROS to induce lasting oxidative stress and the worms were able to compensate and readjust the amount of GSH after depletion. Thirdly, it has been stated that in studies using aquatic organisms it has been shown that changes in antioxidant mechanisms are transient and variable for different species and chemicals (Livingstone, 2001; Barata *et al*., 2005; Cochón *et al*., 2007) and finally, it has been suggested that a number of biomarkers for oxidative stress should be used when investigating oxidative stress in aquatic organisms (Lushchak, 2011). Due to these reasons it cannot be fully concluded that no oxidative stress occurred just because there was no significant change in GSH levels. The results of this study are not in line with ZnO toxicology studies as oxidative stress upon exposure to ZnO has been measured in several human cell lines (e.g. De Beradis *et al.*, 2010; Huang *et al*., 2010; Fukui *et al*., 2012; Sharma *et al*., 2012) and environmental toxicity studies (e.g. Lin *et al*., 2009; Pujalte *et al*., 2011; Hao *et al*., 2012). However, in Hao *et al*. (2012) it was observed that after exposure to ZnO NPs the enzymatic and

non-enzymatic antioxidant defences of carp (*Cyprinas carpio*) acted in different ways in different organs. They suggested that this was proof that in order to fully evaluate oxidative stress biomarkers it is necessary to assess a number of markers in the organism. The evolution of testing oxidative stress markers is key in determining the toxic effects of xenobiotics in aquatic organisms. Verrengia Guerrero *et al*. (2002) suggested however that it may be difficult to interpret responses and body burdens of animals exposed to xenobiotics, due to low concentrations and instability of ROS and ROS induced products and the different responses of various organisms to oxidative stress, leading to difficult evaluation (Lushchak, 2011). This is of particular importance in *L. variegatus* as they are a standard toxicity testing species and if they have detoxification pathways that are different or absent in other species then they may not have a great ecological relevance as previously believed. *In vivo* inhibition or induction of oxidative biomarkers in aquatic organisms is a good ecotoxicological tool to assess the effects of NP exposure (McLouhglin *et al*., 2000) however a battery of markers, for example SOD, CAT, GST and GSH, should be employed to investigate oxidative stress in aquatic organisms.

L. variegatus are capable of ingesting ZnO particles (as determined using STEM-EDX), however, the uptake and depuration protocol employed in this thesis was found to have a high level of variability and it was decided that while this protocol may be appropriate for metals that are not naturally present in relatively large quantities in the environment, it was not suitable for investigating the uptake, bioaccumulation and depuration of ZnO NPs and bulk particles (Table 8.1). Dawson *et al*. (2003) investigated the accumulation of bulk Zn in *L. variegatus* and found that the background levels of Zn in the worm may have affected the result and that variability in the measurements was very high, using ICP-AAS.

Finally, it was concluded from the histology study was that Bouin's solution is an appropriate fixative for fixing *L. variegatus* in a closed processing system. Histology was a useful technique in investigating the anatomical effects of ZnO NPs and bulk particles after a 96 hour exposure (Table 8.1). A change in morphology of the anatomical structure of an organism after exposure to NPs can result in the impairment of functional capacity, recovery capacity or an increased susceptibility to harmful environmental conditions (Fadeel *et al*., 2012). No changes in anatomy were found to have occurred. It is possible that a 96 hour exposure is not a sufficient exposure time to induce visible changes in the worms. It may be that the ZnO NPs were not themselves

visualised either because they had dissolved during exposure and/or as a result of their colour. A longer exposure study would be interesting to investigate whether ZnO induced damage would be observed after chronic exposure. This additional time may allow for any changes to occur within the tissues should a toxic effect be observed. Furthermore, treatment of the tissues with stains other than H and E may (e.g. van Gieson stain, silver stains, acid and alkaline phosphatase stains, Alcian Blue and Oil Red O) potentially provide a greater volume of information in identifying the various structures of the worm. Finally, a positive control, such as boric acid (Martinez *et al*., 2006), could be employed to show damage using histological techniques.

The project included an aim to characterise the ZnO NPs and bulk particles using a suite of techniques at both HWU and FENAC. As mentioned in the previous text this characterisation aided in understanding some of the toxicology results obtained in this thesis. The BET study determined that the surface area of the NPs was significantly greater than that of the bulk particles, suggesting a greater surface area for reactivity in the NPs. This result correlates with the toxicity results observed in the behavioural studies where NPs were found to have a greater negative impact on behaviour than the bulk particles. The dialysis experiment provided information on the dissolution of the ZnO particles and allowed for a greater understanding of whether the mechanism of toxicity in some of the studies was ionic or nano-specific. The use of TEM and STEM-EDX aided in visualising the particles in the medium, medium with HA and within the worms. These images provided information that allowed for a better understanding of the differences between worms exposed to NPs in medium alone and NPs in medium with HA. XRD confirmed that the particles in use were ZnO. Finally, the DLS and zeta potential study (along with the TEM images) provided information on the size distribution and stability of the NPs and bulk particles which helped in understanding the toxicity results obtained in studies by showing that the hydrodynamic diameters of the bulk particles were larger than those of the NPs and also that stability was increased with the addition of HA. Based on the results of the characterisation studies and the information they provided for understanding of the toxicology results, it is clear that a suite of techniques are required in order to fully characterise particle exposures. Understanding and interpretation of the results obtained from the various techniques is paramount.

8.2 Brief summary of current ZnO NP toxicity literature and where the findings of this thesis fit in with this literature

Despite the widespread use of ZnO NPs in industry, the safety of these particles has not yet been fully evaluated. The purpose of this thesis was to provide valid and novel information to contribute to the current literature available on the toxicity of ZnO NPs. Numerous human and environmental models have been examined in order to assess the toxicity of ZnO NPs. In many studies solubility is widely accepted as one of the main factors driving toxicity of ZnO NPs however some studies have stated a nano-specific effect. In human toxicology studies ZnO NPs or their soluble components were found to act by various mechanisms such as high cytotoxicity and apoptosis, inflammation, oxidative stress, fibrosis, up-regulation of enzymes important in the redox balance and metallothionein. Calcium homeostasis, mitochondrial activity and membrane potential, as well as epithelial barrier function were also affected. *In vivo* toxicology studies have indicated that ZnO NPs are toxic when inhaled but do not cross human skin (Vandebriel and De Jong, 2012) and also that ZnO NPs (radioactive) can be found to accumulate in several organs of mice (Chen *et al*., 2010; Li *et al*., 2011). Several inhalation studies (e.g. Warheit *et al*., 2009 (NP effect); Wang *et al*., 2010 (NP effect); Jachak *et al*., 2011 (NP effect)) and instillation studies (e.g. Sayes *et al*., 2007 (NP effect); Cho *et al*., 2010 (ionic effect)) in rats and mice have shown that ZnO NPs cause inflammation, necrosis and systemic toxicity. *In vivo* studies have shown no genotoxic effects of ZnO NPs. *In vitro* studies have stated that ZnO NPs are toxic to several cell types, such as lung THP1 monocytic cells (Prach *et al.*, 2013 (NP and ionic effect), BEAS-2B cells (Sharma *et al*., 2009 (NP effect); Huang *et al*., 2010 (NP effect); Wu *et al*., 2010 (NP effect)), colon carcinoma RKO cells (Moos *et al*., 2011 (ionic effect)), leukaemia cells (Kao *et al.*, 2012 (ionic effect)), cardiac microvascular endothelial cells (Xia *et al.*, 2011(ionic effect)), C3A liver cells (Wang *et al*., 2011 (NP effect); Kermanizadeh *et al*., 2012 (NP and ionic effect)), glomerular measangial cells (Pujalté *et al*., 2011 (NP and ionic effect) and have also exhibited genotoxicity (Hackenberg *et al*., 2009 (NP effect); Sharma *et al.*, 2009 (NP effect); Osman *et al.*, 2010 (NP effect); Sharma *et al.*, 2011 (NP effect)) *in vitro*. However, some studies have stated that not only is the toxicity is down to a nanospecific effect it is also, at least in part, a result of dissolved Zn^{2+} from the dissolution of ZnO NPs (Vandebriel and De Jong *et al*., 2012).

In environmental toxicology studies the ZnO NPs were also found to cause toxicity via various mechanisms. Environmental models, such as plants, algae, microorganisms, fish and aquatic invertebrates have been used to investigate the environmental toxicity of ZnO NPs. In plants ZnO NPs were found to have toxic effects via morphological changes, biomass reduction, growth inhibition, germination inhibition and the up-regulation of enzymes (e.g. Lee *et al*., 2010 (NP effect); Du *et al.*, 2011 (ionic effect); Ghodake *et al*., 2011 (NP effect); Hernandez-Viezcas *et al*., 2011 (ionic effect)). Zinc ions were said to be both major and minor contributors to this toxicity. ZnO appears generally to be phytotoxic however only at ecologically irrelevant concentrations. Only one study has shown toxicity in algae however the authors attributed this toxicity to ionic Zn (Aruoja *et al*, 2008). Bacterial studies described mechanisms of toxic effects as inhibition of growth and cell viability and mortality. In microorganism studies (e.g. Heinlaan *et al*., 2008 (ionic effect); Blinova *et al*., 2010 (ionic and NP effect); Dimkpa *et al.*, 2012 (ionic and NP effect) toxicity was found to be both NP specific and ionic. The media type played a major role in these toxicity results. Finally, invertebrate and vertebrate toxicity studies stated that ZnO toxicity mechanisms included morphological changes, reproductive inhibition (embryo/larvae survival and development), behavioural inhibition and changes, enzyme up-regulation, mortality and the bioaccumulation of particles, via both ionic and NP effects. Fish studies showed significantly reduced embryo hatching and survival rates, abnormalities and decreased survival rates of hatched embryos at higher concentrations. ZnO NPs have also been shown to cause oxidative stress *in vivo* in carp (Hao *et al*., 2012 (NP effect)), zebra fish (e.g. Zhu *et al*., 2008 (NP effect); Johnston *et al*, 2010 (ionic and NP effect); Hu *et al*., 2011 (NP effect)) and *in vitro* in catfish hepatocytes (Wang *et al*, 2011 (NP effect)). Aquatic invertebrates have shown toxic responses to ZnO NPs also. *L. luteola* exhibited toxicity at low doses in terms of oxidative stress and DNA damage (Ali *et al*., 2012 (NP effect)) and *L. stagnalis* feeding behaviour was inhibited after exposure to ZnO NPs (Croteau *et al*., 2011 (ionic and NP effect). Crustaceans, such as *D. magna* (Heinlaan *et* al., 2008 (ionic effect); Wiench *et* al., 2009 (ionic effect); Blinova *et al*, 2010 (ionic effect); Poynton *et al*, 2011 (NP effect)), *T. platyurus* (Blinova *et al*., 2010 (ionic and NP effect) and *C. affinis* (Tomilina *et al*., 2010 (NP effect)) were also seen to be affected, in terms of mortality and reproduction by ZnO NP exposure. Abnormalities and mortality were observed in *L. pictus* (Fairbairn *et al*., 2011 (ionic effect)) after exposure to ZnO NPs. Finally, a number of

nematode studies have shown toxicity to *C. elegans* in terms of larval growth and reproduction and movement in adults (e.g. Ma *et al*., 2009 (ionic effect); Ma *et al.*, 2011 (NP effect)).

The findings of this thesis are comparable with current literature. No studies have been published to date investigating the toxicity of ZnO NPs in *L. variegatus* and so all research conducted with *L. variegatus* in this thesis is novel and will contribute to the current knowledge base concerning the toxicity of ZnO NPs. Acute and chronic behavioural studies showed that ZnO NPs were toxic to *L. variegatus*. Dissolution was taken into account in these studies and the results show that the toxicity may have been in part due to zinc ions however they may also have been exposed to insoluble NPs or agglomerates. The literature has stated that the factor of ionic toxicity when examining the effects of ZnO NPs is of great importance for future research and the findings of this thesis confirm that. This thesis shows that ZnO NPs did not cause a depletion in GSH in the cells of the worms. This was not to be expected as ZnO has been shown to cause oxidative stress in a number of human and environmental models (e.g. Ali *et al*., 2005; De Beradis *et al.*, 2010; Huang *et al*, 2010; Xiong *et al*., 2011). The results of the study would suggest that other markers of oxidative stress should be employed in order to fully assess the potential oxidative stress response of *L. variegatus* to ZnO NPs.

8.3 Future work based on the findings of this thesis

As much as this thesis has answered questions regarding the toxicity of ZnO NPs it has also provided additional questions. As with any toxicological studies it would be interesting to investigate a number of endpoints further. The behavioural endpoint employed in this thesis provided promising toxicological information and it would be interesting to examine further behavioural aspects of *L. variegatus*, such as burrowing behaviour, feeding rates and electro-physical techniques to test the conductivity of neural pathways. It would also be useful to test the chronic effects of ZnO NPs spiked into artificial sediment as this would provide more environmentally relevant information than the paper sediment. Furthermore, it has been suggested that chronic studies will provide more information of greater environmental relevance that acute studies.

The use of a sublethal bioassay to examine GSH content in the cells of *L.* variegatus was successful in this thesis however several markers of oxidative stress in *L. variegatus* could be further evaluated. Of the few papers that have been published to date which have investigated oxidative stress in *L. variegatus*, all have looked at more than one marker, such as CAT, LPO, SOD and GST. It would be interesting to examine these other markers in response to ZnO exposure and also to examine longer time points to see if there was any chronic oxidative stress observed in *L. variegatus* after exposure to ZnO NPs. It would be interesting also to examine whether any DNA damage occurs in *L. variegatus* after exposure to ZnO NPs using a technique such as the comet assay.

The uptake and depuration study in this thesis was not successful due to the protocol not being suitable for use with ZnO. Other options for examining the uptake, depuration and bioaccumulation of ZnO in *L. variegatus* would be to investigate the use of dyes such as Newport Green PDX™ Acetoxymethyl Ether, FluorZin™-3, AM, cell permeant and RhodZin™-3, AM, cell permeant. These dyes, available for use in cell culture, fluoresce in the presence of Zn^{2+} and could potentially be further developed for use with whole organisms. To visualise ZnO particles high resolution X-ray absorption techniques, which can be used to analyse the major element-composition in e.g., biological samples, may be useful. Although the resolution of these techniques is not high enough to localise single nanoparticles, it would be possible to distinguish aggregates of ZnO particles from organically complexed or dissolved Zn, due to their higher concentration of Zn.

The histological studies within this thesis were successful in showing that ZnO NPs and bulk particles did not cause any damage to the tissues of *L. variegatus* as a result of acute exposure. It would be interesting to pursue the investigation of longer exposure times and other stains to visualise various parts of the worm structure better. As discussed in Chapter 2, Section 2.4.2, ZnO particles were found within the gut of the worms (using STEM-EDX). It would potentially be useful to use the stains mentioned above to attempt to visualise where the Zn^{2+} can be found within the worm.

8.4 Final remarks

There are a number of issues that have not been addressed in the current literature with regards to the ecotoxicology of ZnO NPs. Future research should focus on the characterisation of ZnO NPs, the relevant exposure concentrations and the exposure scenarios they are used in. The toxicity of the NPs may be elicited by different modes of action and is very much dependent on the parameters of the medium they are in. A thorough characterisation of these factors is essential in interpreting any toxicological results obtained as well as understanding any comparisons made between studies. Research should also focus on chronic exposure scenarios in order to gain more environmentally relevant information from studies. Finally, research should encourage the development of tools to differentiate between Zn^{2+} toxicity and nanospecific ZnO NP toxicity.

Appendix A: Figures from DLS 96 hour study

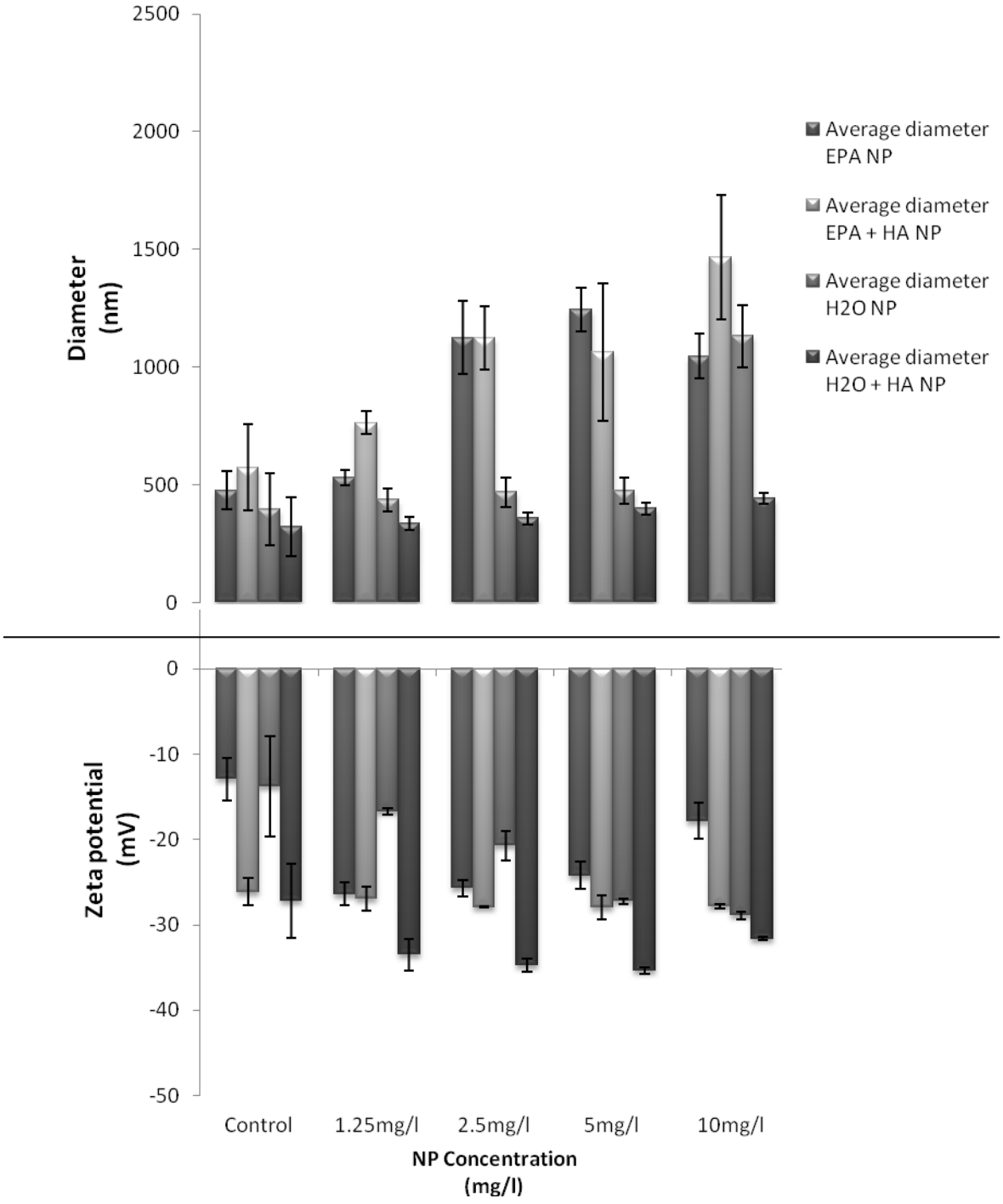

Figure 1: The hydrodynamic diameter and zeta potential of ZnO nanoparticles at 0 hours (mean ± SE; n = 3).

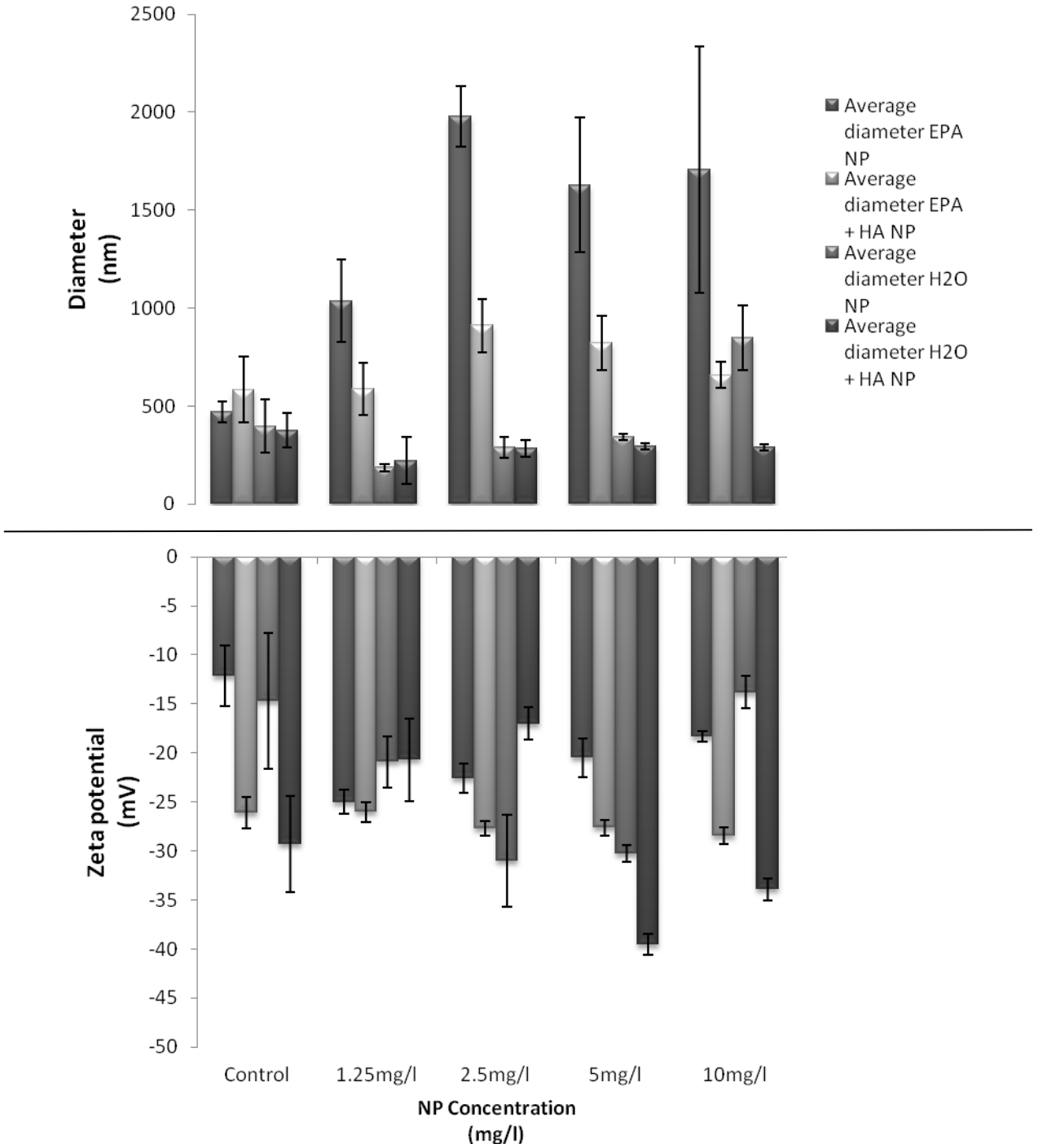

Figure 2: The hydrodynamic diameter and zeta potential of ZnO nanoparticles at 24 hours (mean ± SE; n = 3).

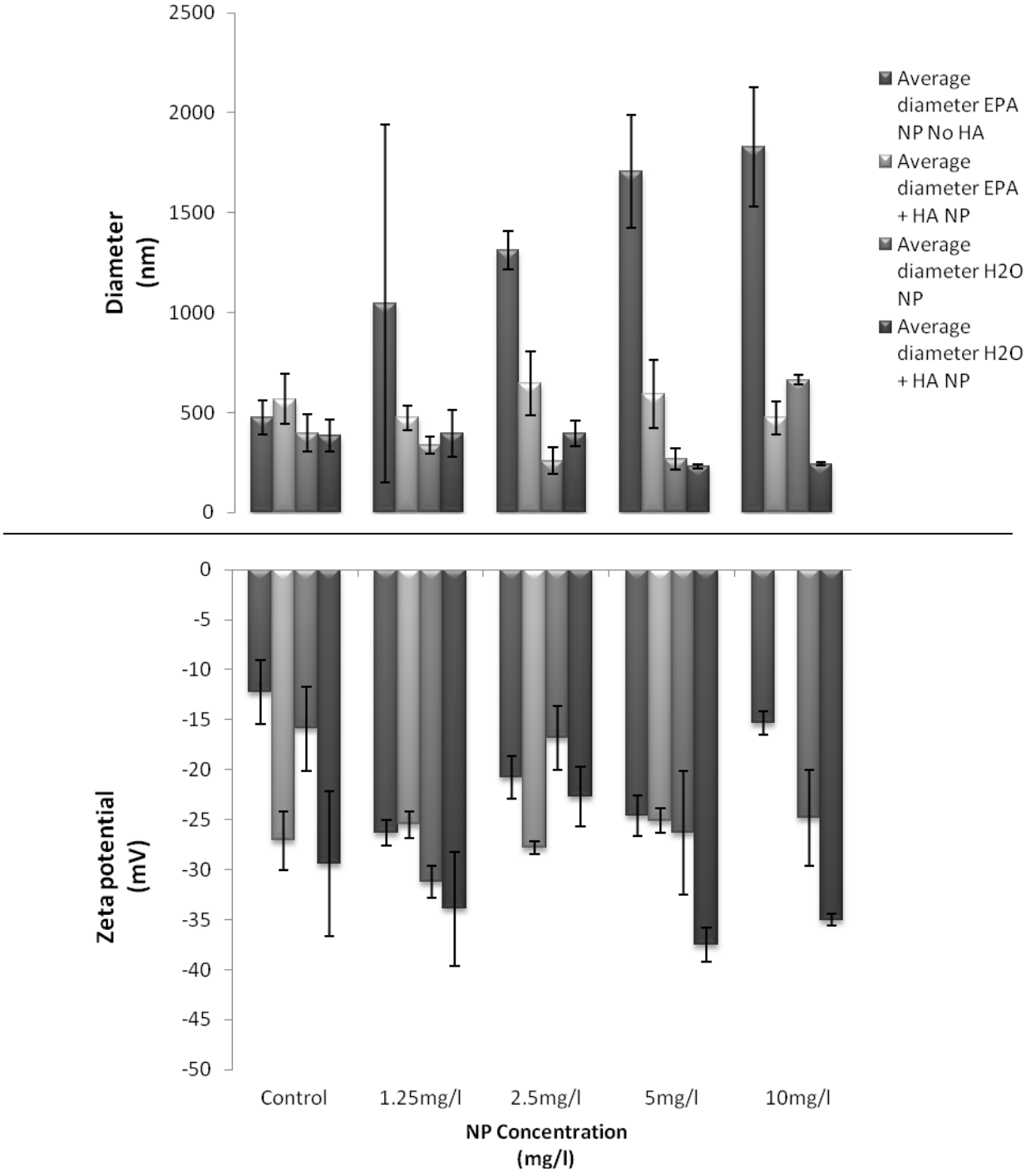

Figure 3: The hydrodynamic diameter and zeta potential of ZnO nanoparticles at 48 hours (mean ± SE; n = 3).

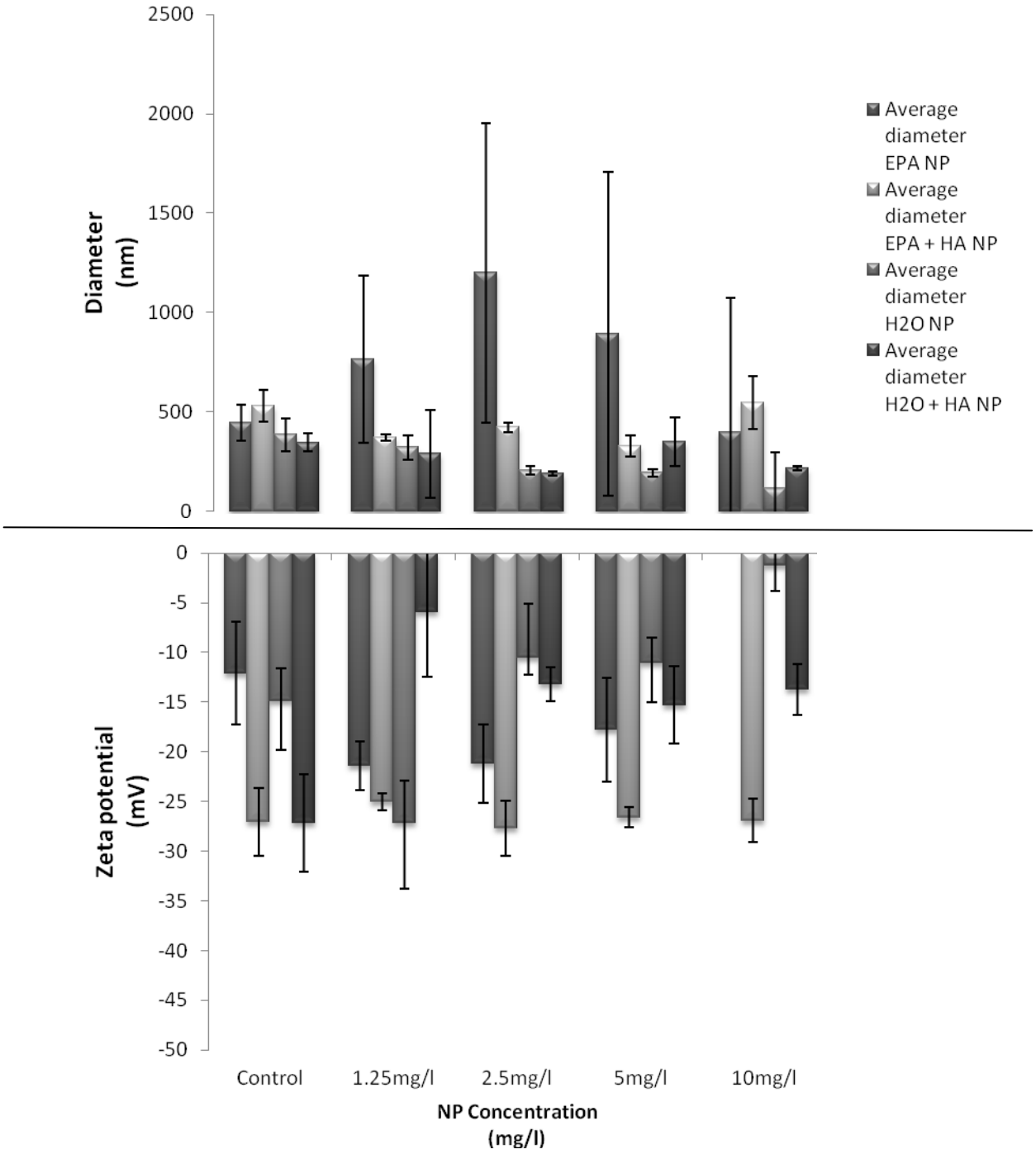

Figure 4: The hydrodynamic diameter and zeta potential of ZnO nanoparticles at 72 hours (mean ± SE; n = 3).

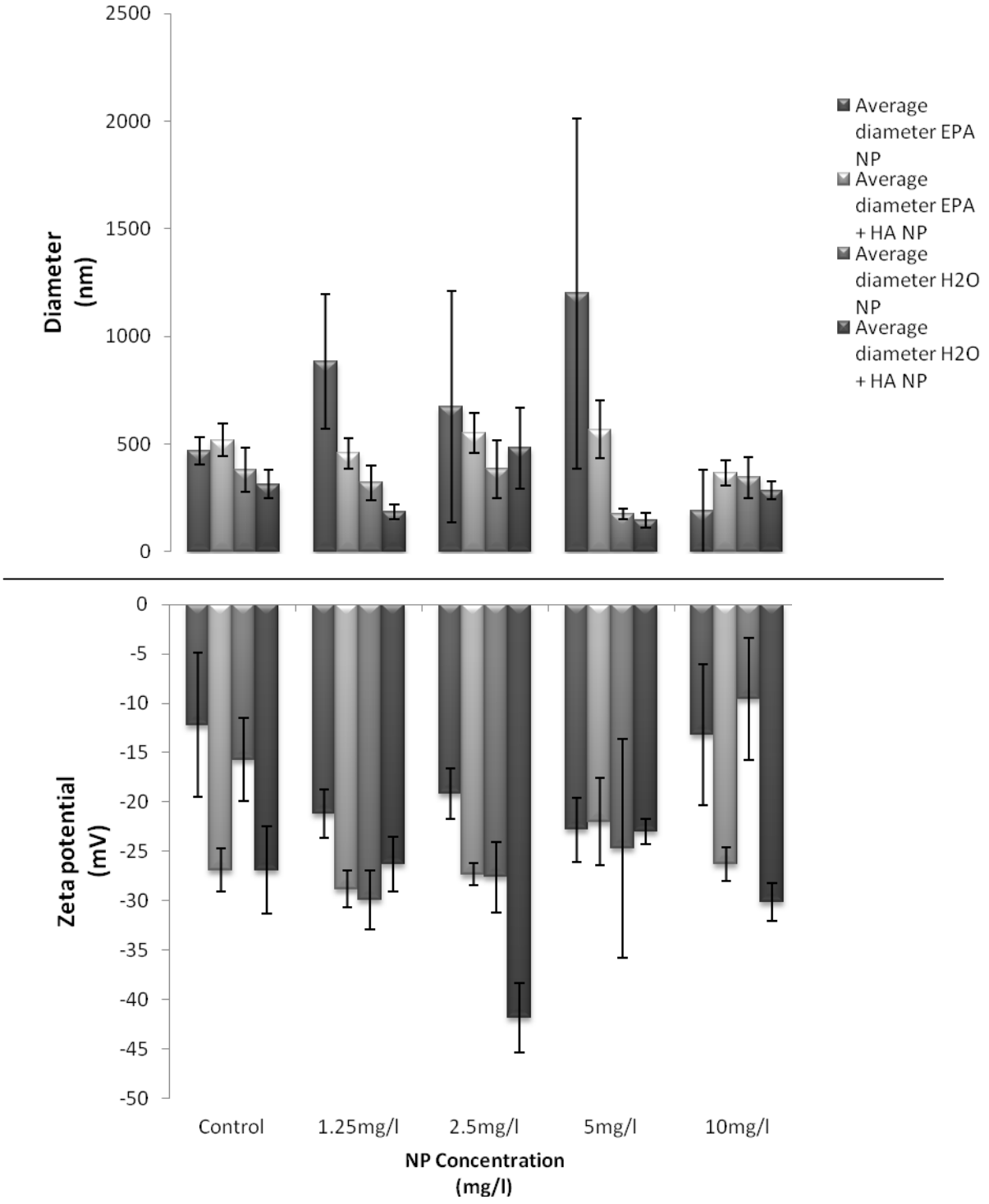

Figure 5: The hydrodynamic diameter and zeta potential of ZnO nanoparticles at 96 hours (mean ± SE; n = 3).

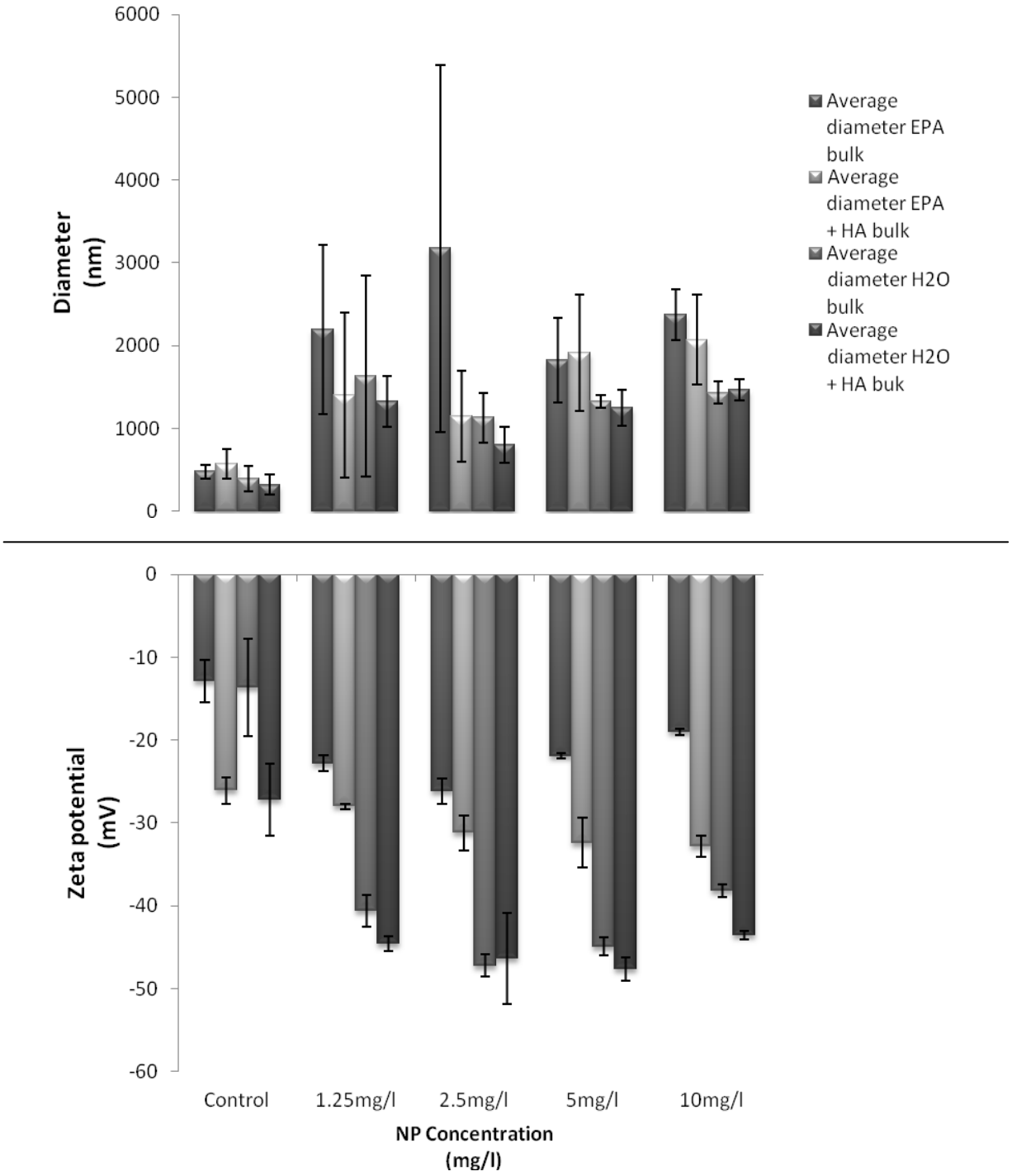

Figure 6: The hydrodynamic diameter and zeta potential of ZnO bulk particles at 0 hours (mean ± SE; n = 3).

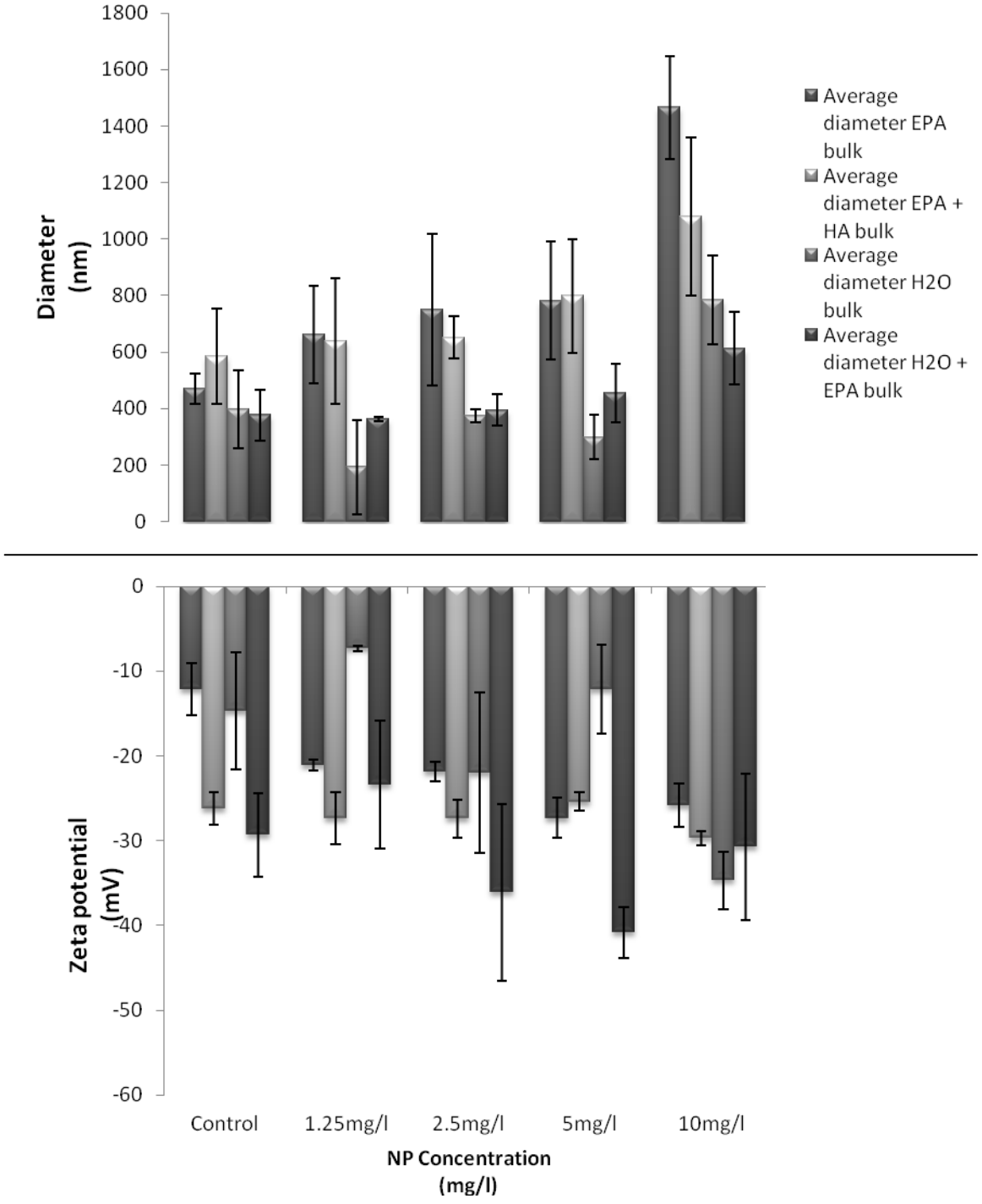

Figure 7: The hydrodynamic diameter and zeta potential of ZnO bulk particles at 24 hours (mean ± SE; n = 3).

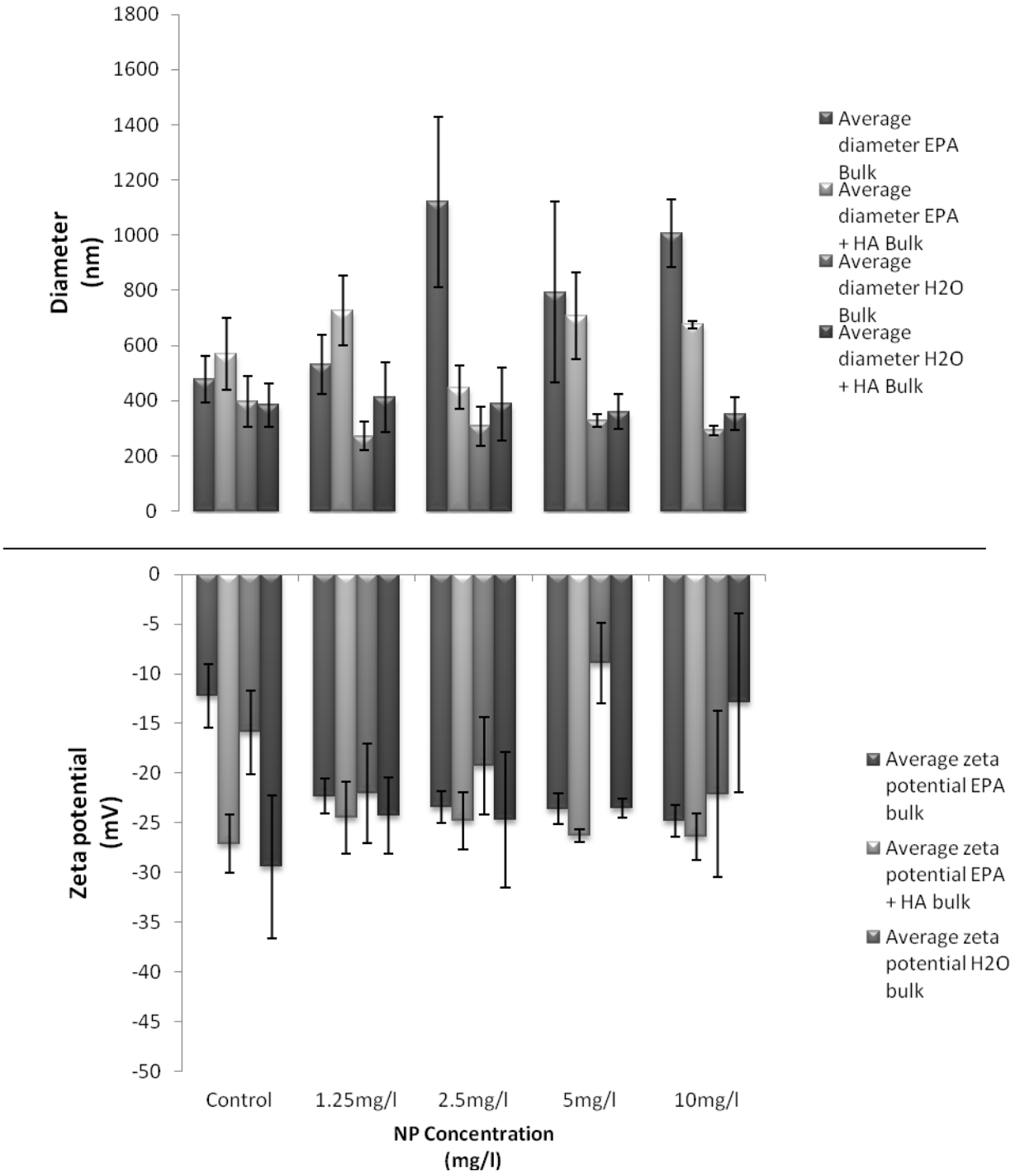

Figure 8: The hydrodynamic diameter and zeta potential of ZnO bulk particles at 48 hours (mean ± SE; n = 3).

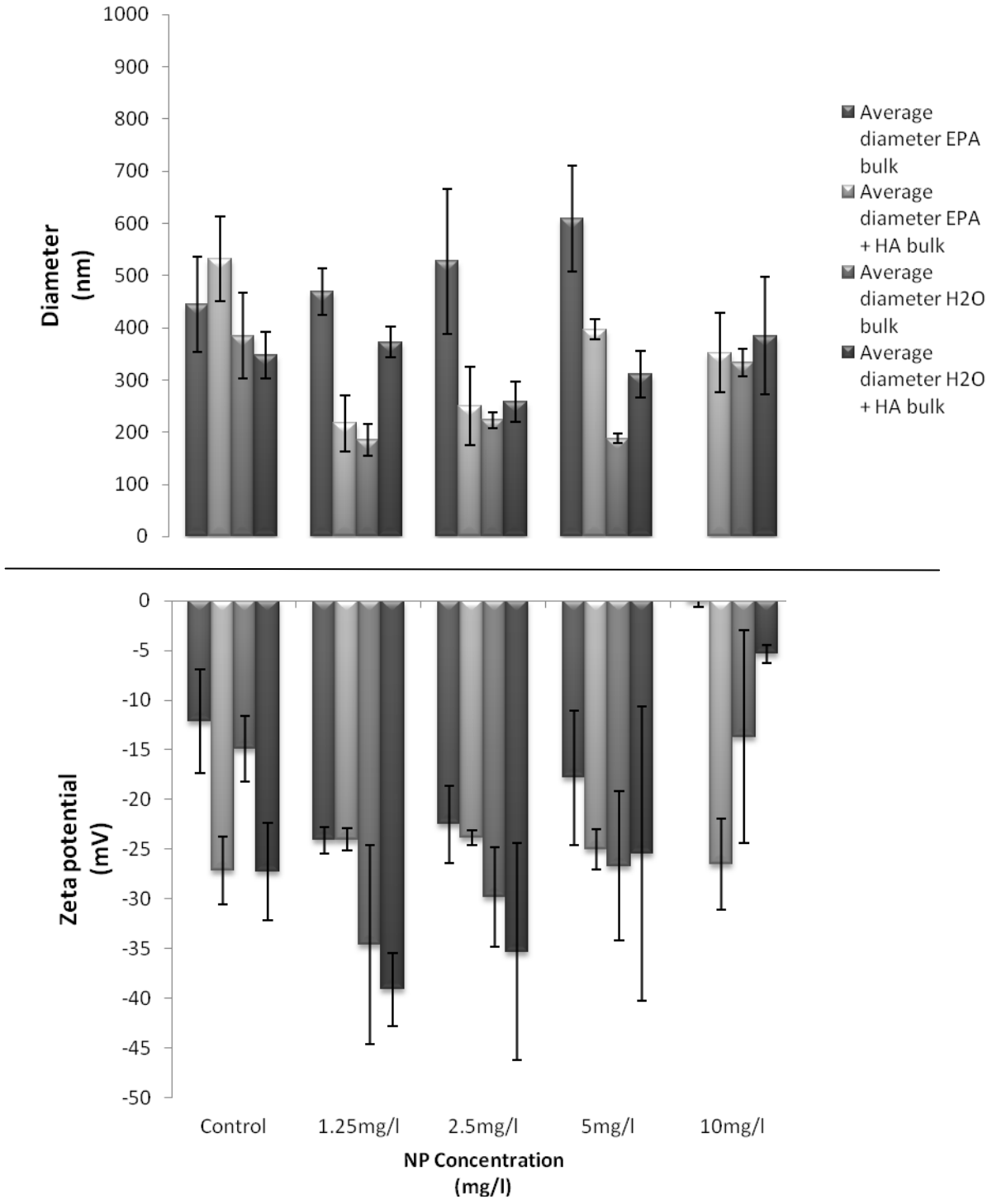

Figure 9: The hydrodynamic diameter and zeta potential of ZnO bulk particles at 72 hours (mean ± SE; n = 3).

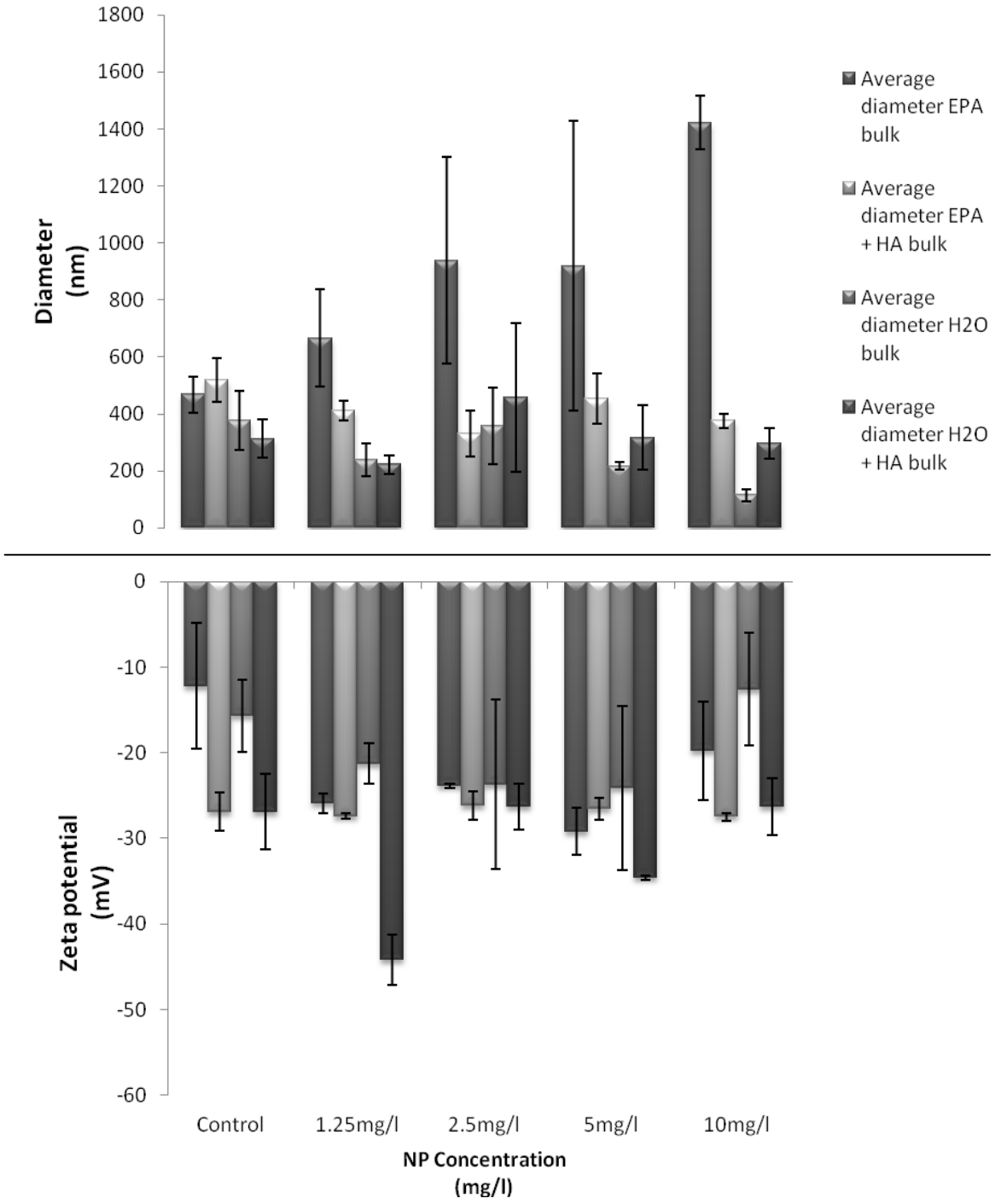

Figure 10: The hydrodynamic diameter and zeta potential of ZnO bulk particles at 96 hours (mean ± SE; n = 3).

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